5619-11

THE AMERICAN NATURALIST

EDITED BY JAQUES CATTELL

A BI-MONTHLY JOURNAL
DEVOTED TO THE ADVANCEMENT OF THE BIOLOGICAL SCIENCES

VOLUME LXXXIV

LANCASTER, PA.
THE SCIENCE PRESS
1950

THE SCIENCE PRESS
LANCASTER, PA.

THE
AMERICAN NATURALIST

VOL. LXXXIV January–February, 1950 No. 814

REACTION OF POPULATIONS OF UNICELLULAR ORGANISMS TO EXTREME CHANGES IN ENVIRONMENT[1]

DR. M. DEMEREC
CARNEGIE INSTITUTION OF WASHINGTON,
COLD SPRING HARBOR, NEW YORK

IT is now an established fact that the cell is the ultimate unit of the genetic system—that, as a rule, each cell possesses a full complement of genetic determinants, genes, which govern its vital physiological processes. A multicellular organism is only an aggregation of units, which theoretically have equal genetic properties, but many of which, particularly at the higher levels of organization, have become adapted to the community interest as it has evolved along with the multicellular condition of the individual. In this process of adaptation, many cells have undergone substantial changes; differentiation has occurred in various degrees to form the aggregates called tissues, to produce organs, and to develop the intricate relations among organs that exist in higher organisms. Essentially, however, the genetic potentialities of each member of the whole colony of cells that constitutes an individual are the same. A very rough parallel to this situation is found in a colony of bees, where eggs that are genetically the same will develop into either queens or workers, depending on the treatment they

[1] Lecture given at the Second Annual Scientific Meeting of the Detroit Institute of Cancer Research, Detroit, Michigan, September 13, 1949. The work was done in part under a grant-in-aid from the American Cancer Society recommended by the Committee on Growth of the National Research Council.

receive, or a colony of ants, where similar eggs will develop into egg-laying females, workers, or soldiers, according to the environmental conditions they are exposed to.

Viewed from this standpoint, many complex biological processes may be more easily understood, and certain experimental results may receive a clearer interpretation. I intend here to analyze the forces that operate in the adjustment of a population of simple unicellular organisms to a radical change in environmental conditions. In illustration of this process I intend to describe the mutual adjustment of bacteria and bacteriophages to each other, and to discuss in some detail our current work on the adjustment of bacterial populations to the presence or absence of streptomycin.

It is well known that the bacteriophages affecting certain bacteria can invariably be found in places where these bacteria are present in great abundance. For example, a strain of phage that is active on any special strain of *Escherichia coli* is most likely to be found in a sample of material from a cesspool, or other water polluted with organic matter, where *E. coli* is present in large quantities. Such material will very probably yield a strain of phage that affects any particular *coli* one is working with, and in addition a number of other strains of phage affecting many other strains of *coli*. It may seem strange that the *coli* are able to flourish side by side with the phages that presumably attack and destroy them. The explanation is found in the experimental work of Luria and Delbrück (1943) and Demerec and Fano (1945), which has revealed that genetic changes in bacteria give rise with great regularity to mutants that are resistant to phages. It is true that the rate at which such mutations occur is exceedingly low—about one per one hundred million; but since the number of *coli* usually making up a population is tremendous, it must always include some individuals that will resist the phage present in the environment. While the phage is destroying the sensitive bacteria of the population, these resistant mutants are unaffected, and have the opportunity to multiply and build up a new

population of resistant individuals. One might expect that when all sensitive individuals had been eliminated the phage, too, would come to an end, since it can perpetuate itself only in sensitive bacteria. Yet we know that this actually is not so, for we always find phage and bacteria together. The step that may be responsible for the survival of phage when resistant mutants take over the bacterial population is revealed by Luria's experiments (1945). He has shown that mutations occur also in phages, and that mutant phages arise that can attack the resistant bacterial forms. Again, the frequency with which these mutations occur is extremely low, but it is high enough so that we can expect such mutants in any bacterial population infected with a phage. This new turn of events will again place the bacteria at a disadvantage, since the mutant phage will now attack them and multiply rapidly. But Luria's work also shows that new mutations, resistant to the mutant phage, may occur in the bacteria, and that the chain of adjustments between coexisting populations of bacteria and phage may thus be continued.

It would seem easy and logical to assume the indefinite progression of such a chain of adjustments. Conditions in nature, however, are not so simple as represented here. A complication is introduced by the fact that several varieties of phage may attack the same strain of bacteria, and that mutants resistant to one variety may still be sensitive to another. This increases the complexity of the situation, but does not rule out the possibility of an explanation. Presumably there is a genetic mechanism that regulates intricate adjustments in the dynamics of coexistent bacterial and phage populations; and the solution of this problem should be a fascinating research, requiring a great deal of skill and ingenuity.

Similar genetic mechanisms are responsible for the origin of bacterial resistance to radiations and to various chemicals, including antibiotics. These mechanisms are capable of initiating changes in the constitution of bacterial populations which, when circumstances demand, will protect

them from extinction. During the past year I have been making an extensive study of the genetic mechanism responsible for the origin of bacterial resistance to streptomycin. This study has revealed several interesting facts bearing on the problem discussed here, and so will be presented in some detail.

The major portion of our work has been done with the B strain of *E. coli*. For some experiments, in which Lederberg's technique of crossing was employed, we used his K-12 strain, since it is the only known strain of *E. coli* with which it is possible to do such experiments.

Several years ago we showed that bacteria resistant to streptomycin originate by mutation (Demerec, 1948). It was found, furthermore, that a single mutational step may be sufficient to account for a change from streptomycin sensitivity to complete streptomycin resistance. Since we worked with a concentration of streptomycin of 25 micrograms per milliliter, complete resistance, for us, means resistance to at least that concentration. Subsequent analysis of 208 completely resistant mutants, which originated independently of one another, showed that in our strain of *coli* about 60 per cent. of such mutants are dependent on streptomycin—that is, require streptomycin for growth. Thus in one mutational change it is possible to obtain, from sensitive bacteria, individuals that require streptomycin in order to grow. For the geneticist, this is a very interesting feature, as it makes possible experiments that can not be made with any other material known so far. By plating bacteria onto medium containing streptomycin, we can isolate resistant mutants from a population of a sensitive strain; and by plating a streptomycin-dependent population onto medium lacking streptomycin, we can detect reverse mutations to non-dependence and sensitivity. In both cases, very large numbers of bacteria can be used: for the study of mutations to resistance, about one billion bacteria per plate; and for the study of reversions, about one hundred million per plate. This use of large numbers makes possible the quantitative determination of exceedingly low rates of spontaneous and induced mutation.

In our experiments we obtained from the B strain of *E. coli* several strains of streptomycin-resistant and -dependent mutants (first-order mutants). From the dependent strains we derived reversions (first-order reversions), and then, in turn, second-order dependent mutants, second-order reversions, and third-order dependent mutants. This procedure was continued through four orders of mutants. All together, 163 streptomycin-dependent and 120 reverse mutants were analyzed in detail. In making the analyses, we studied the following properties:

(*a*) Mutation rate.—This was found to be different in the various streptomycin-dependent and reverse mutant forms. Some mutated at a rate of 5×10^{-8}, others at rates lower than this, and some at a rate lower than 1×10^{-10}.

(*b*) Mutation pattern.—Streptomycin-dependent strains can mutate to either streptomycin-resistant (non-dependent) or streptomycin-sensitive types. The various dependent forms differed in the proportions of resistant and sensitive mutants they produced; some yielded 100 per cent. resistant and no sensitive, some 100 per cent. sensitive and no resistant, and others various proportions intermediate between these two extremes. In the same way, sensitive strains can mutate to either resistant or dependent; and different strains differ in the proportions of these two types they yield—the extremes being 100 per cent. resistant to no dependent, and 40 per cent. resistant to 60 per cent. dependent.

(*c*) Growth rate.—Considerable differences in growth rate were observed among the mutants of both the streptomycin-dependent and the reversion series. Colonies of slow-growing mutants require 48 hours, or even 72 hours, to reach full size, whereas colonies of normal-growing mutants reach this stage in less than 24 hours.

(*d*) Biochemical deficiency.—It was found that some of the reversion and dependent mutants are not able to grow on M-9 synthetic medium, but require full broth medium for their growth.

(*e*) Sensitivity to ultraviolet radiation.—Considerable differences were found between strains with regard to the amount of radiation required to kill them.

(*f*) Streptomycin dependence of dependent mutants.—We were able to test each of the 120 dependent mutants with five degradation products of the streptomycin molecule, as follows:

(1) Tetrahydroanhydrostreptobiosamine hydrochloride.
(2) Streptobiosamine hydrochloride.
(3) Streptidine dihydrochloride.
(4) Streptidine sulfate.
(5) Streptamine dihydrochloride.

We found that degradation products (3), (4), and (5) were not adequate to supply the deficiency in any of our strains. Sixty-six strains could

utilize streptomycin only; forty-four strains were able to grow on (1) but not on (2); nine strains grew on either (1) or (2); and one strain grew on (2) but not on (1). There were also considerable differences in rate of growth among the various dependent strains, on these compounds as well as on streptomycin.

From the results of all these tests together it is evident that hardly any two of the mutants studied by us are alike; and we used only six out of many possible criteria to detect differences among them. This means that a reversion from dependence to sensitivity does not come about by a reversal of the chemical reaction that originally produced dependence, but must be due to a change unrelated to the original one. Since most mutants in this series are different from one another, there must be a considerable number of independent reactions that give rise to either streptomycin-resistant or streptomycin-sensitive mutants.

Let us now for a moment disregard the resistant (non-dependent) mutants and assume that only sensitive and dependent exist. It is true that this situation does not obtain in the streptomycin-resistance system, but it seems very likely that it will be found in some other system involving a chemical or other environmental factor (heat, pressure, age, etc.). Let us suppose that a strain of bacteria, sensitive to streptomycin, is suddenly brought into an environment where streptomycin is present. In this new situation, all the sensitive individuals would be eliminated, but the few streptomycin-dependent mutants that would certainly be present among a large number of bacteria would continue to divide and thus form a new population entirely adjusted to the changed environment. Now let us assume another shift in environmental conditions, such that streptomycin is lost from the medium in which this bacterial population grows. The new environment would eliminate all the dependent bacteria, but would allow streptomycin-sensitive mutants to grow and form a new population.

Thus a strain of bacteria, exposed to extreme changes of

this sort, possesses a very effective mechanism for adjustment and survival. It is evident that such a mechanism is more efficient when the changes in either direction are not limited to a single reversible reaction but may follow several independent paths. It seems to me that in providing a mechanism of this sort nature has furnished better assurance of the survival of a strain and thus of a species.

As a geneticist, I am much interested in learning more about the details of the mechanism of this system of reversible changes to streptomycin resistance. In particular, I am anxious to know whether several gene loci are involved or all the reactions are controlled by a single gene locus. The most direct way to solve such a problem is by determining genetic relationship through intercrossing. Unfortunately, this can not be done with bacteria, at least not with this strain of *E. coli.* Lederberg (1947) has shown, however, in another strain of *E. coli* known as K-12, that if two lines, each carrying several easily detectable heritable characteristics, are plated together, interchanges occur between these sets of characters comparable to the crossing over observed in higher organisms. He has worked out a technique whereby the amount of crossing over can be determined and the distances between loci calculated. If no recombination is obtained between any two of the characters present in the different parent strains, this indicates that the genes responsible for them are either in the same locus (allelic) or in closely adjacent loci.

I have partially repeated with the K-12 strain of *coli* the experiments made with our B strain, and have found very similar behavior with regard to streptomycin resistance. Resistant and dependent mutants occur, the dependent revert to either resistant or sensitive, and the sensitive again give rise to resistant and dependent. Moreover, the various mutants of K-12 show differences among themselves similar to those observed among the mutants of the B strain. Intercrosses involving twelve K-12 dependent mutants and eleven K-12 resistant mutants revealed a very interesting situation. No interchange was observed between

any of them, indicating that all these mutations belong either to the same locus or to adjacent loci. (As far as theoretical considerations are concerned, there is no fundamental difference between these two possibilities.)

In this case a series of reactions appears to be controlled by a single gene locus. A change at this locus may produce a mutant resistant to streptomycin; another change at the same locus, a mutant dependent on streptomycin; another, a resistant slow grower; still another, a dependent biochemically deficient mutant; and so on. It seems as though this particular locus controls a series of interrelated reactions, which may be visualized as a chain, the end result being determined by the point at which the chain is interrupted or broken by a change in the gene—a mutation. This chain may be lineal, or it may be branched—that is, some links along it may serve as starting points for new chains of reactions. Our results indicate that when this chain of reactions is interrupted by a mutation, continuity is re-established (in other words, reversion occurs) not by means of a repair in the interrupted link, but rather through a change in some other link of the main chain or of a side chain, which change re-establishes the broken reaction, or induces the development of a product to replace the one suppressed by the original mutation. At present I am not in a position to elaborate the details of this procedure, but I may be able to do so when analysis of the material we now have is completed.

According to the information now available, this interrelated system of reactions affects the following properties: resistance to streptomycin; dependence either on streptomycin or on some part of the streptomycin molecule; resistance to radiations, mustard compounds, penicillin, and neomycin; some not-yet-identified biochemical deficiencies; mutation rate; and mutation pattern. The system does not include resistance to the seven phages of the T series, however. We have tested about 500 streptomycin-resistant and streptomycin-dependent mutants with phages of the T series, and have not detected any instance of a change in-

volving both resistance to streptomycin and resistance to phages. This suggests either that bacterial cells possess several entirely independent systems of reactions, or that there is only a rare connection between reactions involved in different systems.

Now, permit me to indulge for a few moments in speculation concerning the nature of a gene that could perform the functions required by the accumulated evidence. If we visualize a gene as an organic molecule—which seems to be the only reasonable way to picture a gene—then this molecule must be a complex one, since it must be capable of a number of changes. It must also be differentiated, to account for differences between the results of these changes.

We know that genes are arranged in linear order in a more complex structure known as the chromosome. We have good reason to believe that nucleoproteins and nucleic acids make up the main substance of both chromosomes and genes. A gene may be visualized as consisting of a central core—a thread-like structure, which may be continuous, linking all the genes of one chromosome—and side groups attached to this core. In such case, the side groups could be the sites of potential differentiation, where control of the various steps of reactions occurring in the system resides.

This speculation leads to the question whether it would be possible to estimate the number of differentiating sites (or "side groups") present in a gene locus in bacteria. This is certainly not impossible; in fact, I am hopeful that we may be able to acquire some definite information on the subject. So far, only a set of preliminary experiments has been done, with encouraging results. I shall describe in a few words our experimental approach. As I mentioned earlier, our series of mutants includes some that mutate to a new form at a rate of about 5×10^{-8}, and others that have a much lower mutation rate—less than 1×10^{-10}. This difference in mutation rate may be due to one of two factors: either (1) to a difference in the rate of a single chemical reaction, which is responsible for the mutation; or (2) to a difference in the number of sites at which the

mutational change may occur. In the latter case, a high-mutating strain would have more sites than a low-mutating strain, by a factor approximately equivalent to the difference between their mutation rates.

There is a way to distinguish experimentally between these two possibilities, by comparing rates of mutation induced by irradiation either with X-rays or with ultraviolet rays. We have found that both of these give a linear increase of mutations with increased dosage, approximating a single-hit curve. If the first possibility were the correct one, and the difference between the mutation rates of the two strains were due to differences in the speed of one chemical reaction, then we would expect that similar radiation treatment would induce a similar number of mutants in both strains. If the second possibility is true, however, and the difference in mutation rate is due to a difference in the number of sites at which such mutations may occur, the low-mutating line, presumably having fewer sites, will also yield fewer radiation-induced mutants. This is exactly what happened when the test was carried out. Whereas ultraviolet treatment of the strain showing the higher spontaneous mutation rate produced 6,600 induced mutants, a similar dose applied to bacteria of the low-mutating strain resulted in no detectable induced mutants whatever.

If this reasoning is correct, differences in number of sites at which mutations may occur are responsible for the differences of mutation rate among our strains. It then follows that it should be possible to reduce the number of sites—to lose some—through mutational changes. It also follows that we may use the rate of spontaneous mutation as an indication of the number of sites possessed by a gene or a set of genes. From the experimental evidence available at present, it appears that mutation rate per gene locus (disregarding the highly mutable genes) is considerably lower in bacteria, where the range is between 5×10^{-8} and 1×10^{-9}, than in *Drosophila* or in man, where the range is between 1×10^{-5} and 1×10^{-6}. From this it would appear that genes in higher organisms are more highly differentiated

than genes in bacteria, which in turn would make it seem probable that the difference between lower and higher organisms is not in the number of gene loci they possess but in the degree of organization of these loci. Such an interpretation would make it easier to account for the fact that a bacterial cell is biologically as complex as cells of a higher organism, and its requirements for essential compounds not much different from theirs.

Before concluding, I should like to discuss briefly another problem related to the concept emphasized in the early part of my talk. There it was mentioned that the cell constitutes the unit of the genetic system, and that a multicellular organism is composed of a number of cells, equal in genetic potentiality, which are controlled by the yet-uncomprehended forces concerned with development. Still, in multicellular organisms the genetic mechanism of individual cells is constantly changing—mutating; and moreover the environmental factors affecting the cells in which such changes occur play a considerable role in determining their future. From all that is known at present, we may state with reasonable certainty that among the mutations occurring in the somatic cells of a higher organism there are some that change the properties of a normal cell and make it function as a cancerous cell. Data analyzed by Haldane (1949) indicate that the genes in man mutate with an approximate frequency of 2×10^{-5}. Since a human body contains about 1×10^{13} to 1×10^{14} cells, it would be expected—if there were only one gene locus for cancer—that the gene determining cancer would mutate in about 2×10^{8} cells, and that the mutation would be present in many more cells (about 6×10^{9}) through division of those in which it had occurred. Since somatic cells are diploid, and it is likely that the cancer mutation is recessive, it would be necessary for it to become homozygous in order to be expressed. Taking into account the number of cells in the human body and the frequency with which mutations to cancer probably occur, one might expect that on the average about 100,000 such homozygous cells would be formed

through mutation in a human body, and that each of these could be considered the potential focus of a cancerous growth. This raises the question why cancer is not more prevalent than it is, and how some people escape it. The most likely explanation is the effect of environmental factors on newly originated cancerous cells. Such a cell must compete for survival with the normal cells adjacent to it; and, since a cancerous cell usually is different from a normal one in metabolic and physiological activity, it might easily be at a disadvantage, and so be suppressed in its growth and eventually eliminated. Judging from the incidence of cancer, only a small fraction of the original number of cancerous cells is able to develop and cause trouble.

I shall conclude by emphasizing again the importance of the concept, first, that a cell is a unit of organized life, and second, that every cell, both in unicellular and in multicellular organisms, possesses the genetic capacity of the species to which it belongs.

LITERATURE CITED

Demerec, M.
1948. *Jour. Bacteriology,* 56: 63–74.
Demerec, M., and U. Fano
1945. *Genetics,* 30: 119–136.
Haldane, J. B. S.
1949. *Proc. 8th Intern. Congress of Genetics* (Stockholm, 1948): 266–273.
Lederberg, J.
1947. *Genetics,* 32: 505–525.
Luria, S. E.
1945. *Genetics,* 30: 84–99.
Luria, S. E., and M. Delbrück
1943. *Genetics,* 28: 491–511.

MEIOSIS IN AN UNUSUAL FORM OF *ACONITUM UNCINATUM* L.

DR. HENRY W. JENSEN
WARREN WILSON COLLEGE, SWANNANOA, N. C.

FOUR years ago the author's attention was called to a patch of wild monkshood or wolfbane, growing on a steep roadside bank near the junction of Bull Creek with the Swannanoa River in Buncombe County, North Carolina. The first peculiarity noticed was the variation in flower color—from velvety deep blue through shades of light blue to greenish white. The blue color and the growth of the stems from small tubers suggest that these plants are *Aconitum uncinatum* L. However, the stems are up to eight or nine feet, trailing, with thin leaves; the flowers have a distinct beak, are soon horizontal on the tips of small side branches on the upper third of the stem; and the follicles have slender divergent beaks. These are characteristics of *A. reclinatum* A. Gray. One might suspect these plants to be of recent hybrid origin, although no other stations for *A. uncinatum* were found in the vicinity and the nearest station for the normal white-flowered type of *A. reclinatum* known to the author is about three miles west and 1500 feet higher in elevation. Because the blue color and the presence of the tuber instead of a rhizome are definitely characteristics of *A. uncinatum,* the plants in question will be referred to as belonging to this species.

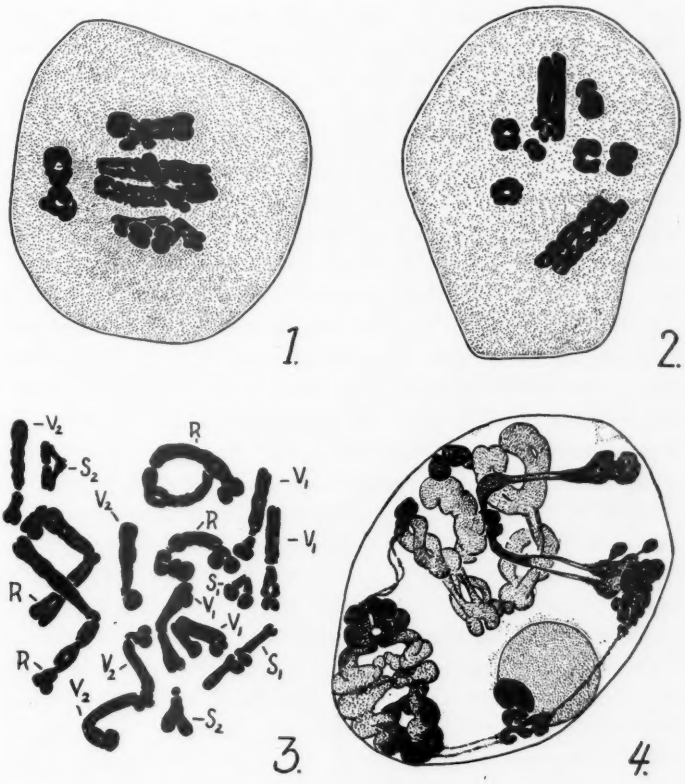
A cytological investigation was undertaken to inquire as to the origin of this particular form of *A. uncinatum.* Collections made during the last week in August and the first week in September, 1946, did not provide good material. A long dry period had adversely affected meiosis. The first or heterotypic division of the chromosomes in microsporogenesis was highly irregular in some anther sacs. Very

often some chromatin was left in the cytoplasm by the end of that division. The second division was also irregular and the resulting microspores were variable in size and displayed a high percentage of abortion. Such cytological phenomena are typical of known hybrids and have long been used as diagnostic of a hybrid origin for natural varieties exhibiting them. Although fixation was not up to standard, the presence of chromosomes in the cytoplasm in the PMC's containing the daughter nuclei of either division is evidence enough that the meiotic irregularities were of natural origin, even if abnormal. Later observations revealed that little seed was present in the follicles that autumn.

In August, 1947, collections were again made. Rainfall had been more normal and this time there was no evidence of dehydration. Meiosis in this material proceeded normally, there being no laggards, no extruded chromatin, and no extra nuclei. The pollen was structurally perfect.

Of interest is the frequent "secondary association" among the eight pairs of haploid chromosomes, resulting in what often appears to be only four pairs of chromosomes, as shown in Fig. 1. However, the eight chromosomes are easily identified in other PMC's, such as that shown in Fig. 2, or the somatic number of sixteen, as shown in Fig. 3. In fact, this plant provides a rather good object for research on the origin of meiotic chromosomes because of their good size, low number, and the absence of extraneous substances in the cells which would absorb the stain and interfere with the clarity of the chromosome features.

Among the meiotic chromosomes, the larger are two pairs of quite identical ring chromosomes, each with an arm. When the ring chromosome pairs were associated, it was usually through side-by-side contact of the arms. Among the somatic chromosomes, those marked with R in Fig. 3, the four long chromosomes possess a longer and a shorter arm. Returning to meiosis, the next in order of size are two associations of V-shaped chromosomes of almost identical length. Their somatic equivalents are marked V_1 and



FIGS. 1–4. *Aconitum uncinatum* L. 1. Polar view of heterotypic division of microsporogenesis showing association of two sets of chromosomes. 2. Similar stage in meiosis but showing the eight haploid chromosomes without association. 3. Somatic metaphase showing diploid number of chromosomes, 2n—16, with individual chromosomes catalogued. 4. Late meiotic prophase showing the origin of the associations, and the order in which the distinct types of chromosomes arise in the spireme. Note the connecting strands between the chromosomes.

V_2 in Fig. 3, there being four somatic chromosomes of each type. In the V_1 chromosomes the shorter arm is about two-thirds the length of the longer. In the V_2 chromosomes, the shorter arm is one-third the length of the longer. Finally,

a small chromosome pair and a very small chromosome pair are sometimes associated in the meiotic metaphase. Their somatic equivalents are labeled S_1 and S_2, respectively. Thus, of the eight haploid chromosomes (chromosome pairs) there appears to be two sets of four. The three larger chromosomes have duplicates in the other set, namely, the ring and the two larger V's. The very small chromosome is only present in one set. In the somatic complement there are four R's, four V_1's, four V_2's, two S_1's, and two S_2's. According to the findings of Shafer and La Cour (1934), the presence of long-medium chromosomes and the absence of trabanted chromosomes distinguishes species in the section *Lycoctonum* from the Eu-Aconites in the genus *Aconitum*. Asa Gray put *A. reclinatum* in the section *Lycoctonum*. On cytological grounds, *A. uncinatum* would appear to belong there also. To a degree this particular variety of *A. uncinatum,* possessing some characteristics of both species, indirectly establishes the close kinship of these species as well as their position in the *Lycoctonum* section of the genus.

In view of the frequent "double association" seen at meiotic metaphase, the author wondered about how and when it first takes place. Fig. 4 displays the typical situation as seen in PMC's during late prophase. It is to be noted that the ring, the V_1, and the V_2 associations are present and connected. The connection, not only between identical chromosome pairs in the association, but between the various associations, is often seen to be a double thread. The ring chromosomes seem to be the center of the catenation with the "association" of the larger V-chromosomes following along one arm of the spireme. The ring association is most often found close to, but not attached to, the nucleolus. As the prophase matures, the spireme connections weaken and by so-called diakinesis only a few of the chromosomes may be linked together. On the other hand, the earlier stages in the prophase give one the impression of complete catenation, no visible free ends of chromosomes, although the identity of the individual chromosome can not

be determined among the series of double loops. Thus, from observation it appears that the meiotic chromosomes are thoroughly linked together in early prophase, usually linked together by the time the identity of the chromosomes can be assured, and usually free from one another by the end of the prophase. It is somewhat difficult to conceive of an explanation for the order of the chromosomes in the spireme and the fact that they are connected by double threads in a chain according to the conventional theory of chromosome pairing. The arrangement of the associations and their being linked together is so unnecessary to the synaptic theory. However, according to E. C. Jeffrey (1947) there is no essential difference in the origin and division, except in size and number, of somatic, gametic, and meiotic chromosomes. The author's observations of the behavior of the chromosomes of *A. uncinatum* appear to support Jeffrey's contention.

Shafer and La Cour (1934) as well as Darlington (1937) regard the basic number of the genus Aconitum to be 8—those having 16 somatic chromosomes as being diploids. However, from a study of this variety of *A. uncinatum,* the basic number seems to be 4, because of the presence of two sets of haploid chromosomes, one set being 75 per cent. duplicated in the other. This fact indicates that *A. uncinatum* is a tetraploid. Shafer and La Cour probably regard such species as diploids, because no species has so far been reported with a 2n number of 8. However, in support of 4 as the basic number is the occurrence of 12 as well as 16 chromosomes in some species of Aconitum (Gaiser, 1930). If 8 is the basis number, all polyploids should be multiples of that number, whereas they are multiples of 4.

The presence of secondary association would suggest to some cytologists that this form of *A. uncinatum* is an autotetraploid. On the other hand, the fertility of the species and the marked variability of flower color suggest that it may be an allopolyploid, or amphidiploid, according to the criteria set up by Stebbins (1940). It is the author's opinion that when the slopes of the Southern Appalachian Moun-

tains are more thoroughly explored, a number of intermediate forms between and including the present species of *A. reclinatum* and *A. uncinatum* will be found. More than likely they will all be tetraploids (n—8), but it is also quite possible that a form with the basic number (n—4) may be located. Sometimes the basic types have persisted in the isolated coves of the mountain region, such as in the instance of *T. clavatum* in the highly polyploid genus, *Thalictrum,* Jensen (1943).

LITERATURE CITED

Darlington, C. D.
1937. "Recent Advances in Cytology," P. Blakiston's Sons and Company.

Gaiser, L. O.
1930. Chromosome Numbers in Angiosperms II. *Bibliographia Genetica* VI.

Jeffrey, E. C.
1947. *Science,* 106: 305–308.

Jensen, H. W.
1943. *Bragantia,* 3: 199–222.

Schafer, B., and L. La Cour
1934. *Annals of Botany,* 48: 693–713.

Stebbins, G. L.
1940. Amer. Nat., 74: 54–66.

THE TIME INTERVAL BETWEEN DETERMINATION AND DIFFERENTIATION OF WINGS, OCELLI, AND WING MUSCLES IN THE APHID *MACROSIPHUM SANBORNI* (GILLETTE)[1]

DR. JAMES B. KITZMILLER

DEPARTMENT OF ZOOLOGY, UNIVERSITY OF ILLINOIS

ONE phase of the analysis of developmental problems deals with the processes known as determination and differentiation. The investigations of experimental embryologists have demonstrated that in some animals the fates of tissues and structures are irrevocably fixed before the first cleavage of the zygote. That is, portions of the egg are already determined in the sense that they will produce certain structures and no others. In other animals, development is indeterminate for a time, which is to say that the potencies of the developing egg are not fixed until a later time. Sooner or later, however, in all forms, there comes a time after which interference with the developmental process can no longer elicit a different response from the embryo. Such a time is spoken of as the time of determination. As usually employed, the term "time of determination" refers to a particular structure or tissue. We might speak, for example, of the early determination of the lens of the eye.

The time of differentiation of a structure, however, refers to that time in development at which a visible morphogenesis takes place. For example, early in embryonic life, the eyes of insects are merely undifferentiated sections of the hypodermis in the head region. Later, under some developmental influence, the hypodermis thickens and grows,

[1] Part of a study carried out in the Department of Zoology, University of Michigan, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

ommatidia form, and the characteristic kind of eye appears. The initial thickening of these cells is referred to as the beginning of visible differentiation.

The processes of determination and differentiation are of course related. Once a structure has been determined, differentiation is inevitable unless artificially checked. The process by which this differentiation takes place, however, is almost entirely unknown. Any evidence which bears upon this process might contribute, ultimately, to its explanation. One type of evidence gained from experiment concerns the rate at which differentiation takes place.

The length of time elapsing between determination of a structure and its differentiation must have some relation to the extent and nature of the latter process. The speed of alteration can hardly be independent of the events involved.

Whether an aphid is to be winged, for example, is presumably determined by its genotype in response to stimulation by environmental conditions.[1] If the time of embryonic determination can be ascertained, and the time measured before the wings are visibly differentiated, then one feature of the process that results in wing production can be observed, namely, how long it takes for the agent, whatever it may be, to act.

It has been demonstrated (Shull, 1928) for one species of aphid, *Macrosiphum solanifolii,* that the mean time of determination of wings is about 34 hours before birth. In this same aphid, the mean time of differentiation of wings is about twelve hours later. Visible differentiation in this form follows very closely upon the event which irrevocably determines that there is to be such differentiation. The developmental response occurs rapidly. In another species of aphid belonging to the same genus, *Macrosiphum sanborni,* however, White (1946) in the course of an entirely different investigation mentioned that she was unable to detect wing thickenings in the unborn embryos. This suggested the possibility that wings might be differentiated much later than in *M. solanifolii,* and if so, the time between determination and differentiation might be considerably greater.

The present study was undertaken to find out whether this were indeed the case, and to determine the period of time which elapses between determination and differentiation of wings in *M. sanborni.* A difference in the duration of this time might offer clues as to the nature of the process. Perhaps such a difference might indicate a different causative agent, or a difference in the mode of its action. Conceivably it might suggest differences in intermediate causative factors, or a longer or shorter chain of events which ultimately caused differentiation. If no significant difference were found, a comparative study of the process in two closely related forms would be of value.

Acknowledgments

The author wishes to express his gratitude and indebtedness to Professor A. F. Shull, under whose direction the work was done, for constant help and encouragement. His appreciation is also expressed to Mr. Mack Newkirk, director of the Botanical Garden of the Toledo Zoological Society, and to Mrs. F. C. Blanchard, director, and Walter Kleinschmidt, supervisor, of the University of Michigan Botanical Garden, for assistance.

Stocks

Macrosiphum sanborni (Gillette) is the small brown aphid found on chrysanthemum plants in gardens and in greenhouses. It was first described by Sanborn (1904) under the name *Macrosiphum chrysanthemi.* Gillette (1908) showed that the specimen described by Sanborn was not *M. chrysanthemi* but a new species to which he gave the name *Macrosiphum sanborni.* Del Guercio (1911) described this species, but placed it in a new genus, *Macrosiphoniella;* many subsequent authors place *sanborni* in this latter genus. Others, notably Hottes and Frison (1931) and Patch (1938), continue to use *Macrosiphum* as the generic name. The validity of *Macrosiphoniella* is beyond the scope of this paper; for references concerning the synonymy the reader is referred to Gillette (1908), del Guercio (1911), Theobald (1926) and Patch (1938).

Macrosiphum sanborni has been described by various authors as existing in both apterous and alate parthenogenetic forms, but gamic individuals have not been recorded. In the course of the present study both apterous and alate parthenogenetic forms have been produced, but no bisexual reproduction has been observed.

As White (1946) pointed out, the ordinary condition for *Macrosiphum sanborni* found in nature seems to be winglessness. Both winged and wingless forms were collected from natural situations, however, and it is therefore certain that at least some individuals are winged even without treatment in the laboratory. How great this percentage is, or whether it is related to seasonal changes, is not known. Wings can be produced in the laboratory under the influence of certain environmental conditions, chiefly continuous light and low temperature. A short summary of White's results is given in the following paragraph.

Highest percentages of winged offspring were obtained from parents which had been raised at 12 degrees Centigrade and in continuous light, but whose parents had come from a stock raised at 18 degrees Centigrade in continuous light. Thus a "shock" from 18 degrees Centigrade to 12 degrees seemed to be one of the causative factors in wing production. Stocks maintained at room temperature under ordinary conditions of light and darkness gave only a small (0.6) percentage of winged offspring. Furthermore, stocks kept at 18 degrees did not produce as high a percentage of winged offspring as did the aphids changed from 18 degrees to 12 degrees. The average, even for the optimum conditions, was only about 36 per cent. winged offspring.

From the beginning of the current experiments, it was evident that the strain of *Macrosiphum sanborni* used for the present study behaved quite differently from that used by White. Numbers of offspring with wings were considerably higher at both 18 degrees Centigrade and at room temperature. Particularly fortunate was the increase shown in the 18-degree, continuous-light stock. Aphids maintained by White under these conditions produced only 8.6 per cent.

winged progeny; in the present strain these conditions regularly produced more than 34 per cent. winged offspring. These higher figures are of interest in indicating a difference in the strains used; they also resulted in a considerable saving of labor, since the necessity of a change to colder conditions was eliminated. As the time of determination was found by changing parents from wing-producing to wing-reducing conditions, the higher percentage of winged offspring in the wing-producing conditions made more certain the effect of the change.

Methods

Control of light and temperature: All aphids, except those raised under room conditions, were raised in large constant-temperature cabinets. Adult wingless females were placed on chrysanthemum plants which were then covered with lantern globes and placed upon shelves inside the cabinets. One substrain was kept in continuous light at 18 degrees Centigrade and another was kept at room temperature and in ordinary daylight and darkness. Three 200-watt Mazda bulbs provided the illumination in the lighted cabinets. Attempts to keep a substrain in the dark at 20 degrees Centigrade were not successful. The numbers of young produced, and the rate at which they were produced, were approximately the same for periods of less than ten days, in both the light and the dark. That is, if females remained in 20 degrees Centigrade and the dark for less than ten days, they produced as many young, at about the same rate, as did those kept at 18 degrees Centigrade in the light. When females were kept in 20 degrees Centigrade and darkness for longer periods (10–30 days) they either died or produced very few offspring. Reasons for this effect are not clear; presumably the results can not be attributed to the effects of the darkness upon the plants since the parents were changed to fresh plants daily.

Microtechnique: Specimens were fixed in Bouin's fluid, dehydrated with dioxane, and embedded in tissuemat. Sections were cut at 10 micra and stained with Harris's haema-

toxylin and eosin. For fuller description of these methods see Kitzmiller (1948).

Time of Determination of Wings

In the measurement of the time elapsing between determination and differentiation of wings it was obviously necessary to know the time at which both events occurred. Shull (1928) showed that in *Macrosiphum solanifolii* determination of wings took place on the average about 34 hours before birth, but there was some reason to think that this figure would not be correct for *M. sanborni*. Hence it was necessary to ascertain the time at which wings were determined as well as the time at which they were differentiated.

Under ordinary conditions of light and temperature, winglessness is the usual condition for *M. sanborni*. White found only 0.6 per cent. winged individuals among 470 aphids raised at room temperature and in ordinary daylight and darkness. In the present study, of a total of 447 aphids raised under the same conditions, 49 individuals or 10.9 per cent were winged.

As noted on page 26 under "Stocks," the conditions which White found most favorable for wing production in this species were continuous light and 18 degrees Centigrade. These conditions were repeated in the present study and under their influence wingless females produced 3,441 young of which 1,191 or 34.6 per cent. were winged. Conditions which White found unfavorable for wing production, higher temperatures (20 degrees Centigrade) and complete darkness, were repeated and again proved to favor winglessness. The wing-reducing conditions, however, did not result in complete winglessness in all families. Although the numbers of individuals with wings became steadily less as the parents remained in darkness, nevertheless a few winged individuals were produced as long as seven days after the change from wing-producing conditions. The most logical means of accounting for these seemingly aberrant individuals is that the conditions which tended to reduce wings were not absolutely effective, and that some few individuals

were not affected by them. Perhaps these individuals possessed a higher threshold for the "determiner," or were otherwise different from the other aphids. Higher temperatures and total darkness, however, did result in offspring more than 98 per cent. of which were wingless.

Since conditions were known which would promote wing production, and other conditions known which would restrict it, the time of determination could be ascertained. Wingless females were allowed to remain at 18 degrees Centigrade for a certain period, the percentage of winged offspring noted, then the parents transferred to 20 degrees Centigrade and total darkness. The length of time required before no (or very few) winged individuals appeared among the offspring would then serve as a measure of the time of determination of wings, since all wings had presumably been determined by the wing-producing conditions. Thus if wing determination took place, for example, three days before birth, few winged offspring should be born later than three days after the change.

Accordingly, experiments were begun in which several wingless females of the same substrain were placed upon a plant, put into 18 degrees Centigrade and continuous light, and allowed to remain for a recorded length of time. After several days the parents were transferred to a new plant, and then placed in the 20-degree, dark cabinet. Parents were transferred to a fresh plant daily, and replaced in 20 degrees, darkness, for a total, in most experiments, of six days. With the exception of a few minutes each day, during the process of transfer to a fresh plant, these aphids remained in total darkness. All young collected daily were raised at room temperature and under ordinary conditions of daylight and darkness. Thus were collected a group of offspring born at 18 degrees Centigrade and in continuous light, and successive groups born one, two, three, four, five and six days later. These latter progeny were all born after the change from wing-producing to wing-reducing conditions. All offspring were kept at least eight days, so that winged and wingless individuals were easily distinguished.

TABLE 1

TIME OF DETERMINATION OF WINGS. NUMBERS OF WINGLESS AND WINGED OFFSPRING BORN TO WINGLESS FEMALES UNDER THE ENVIRONMENTAL CONDITIONS NOTED. TOTALS AND PERCENTAGES AT BOTTOM OF EACH COLUMN. C = CONTROL

At 18 degrees Cent. continuous light				On successive days after change to 20 degrees, dark													
				1		2		3		4		5		6		over 6	
Exp.	WS	WD	% WD	WS	WD	WS	WD	WS	WD	WS	WD	WS	WD	WS	WD	WS	WD
21 C	96	34	26.1														
22 C	28	17	37.7														
27	34	44	56.4	15	1	19	1	21	0	2	0	1	0	10	0	45	0
29	140	123	46.7	49	34	3	2	20	7	4	0	9	0	0	0	17	1
30	9	19	67.8	4	7	7	8	15	12	17	4	30	5	20	0	37	1
32 C	24	18	42.8	Before change of experimental females to 20 degrees dark													
	59	24	28.9	After change													
33	56	35	38.4	11	7	5	5	2	0	26	4	19	0	28	2	0	0
34	122	69	36.1	15	4	49	5	12	0	55	0	2	0	32	0	3	0
35	151	55	26.7	0	0	46	9	0	0	3	0	25	0	27	0	91	1
36 C	120	70	36.8	Before change													
	110	125	53.2	After change													
37	158	34	17.7	24	7	13	5	17	2	50	1	0	0	25	0	8	0
38 C	100	70	41.1	Before change													
	48	45	48.4	After change													
38a	0	24	100.0	6	17	5	14	3	6	2	0	6	0	13	0	0	0
39	80	2	2.4	45	4	34	11	44	2	44	2	34	0	56	0	24	0
42	88	77	46.6	0	0	51	76	24	39	36	16	5	1	81	0	0	0
43 C	140	61	30.3	Before change													
	17	48	73.8	After change													
44	23	19	45.2	25	8	77	46	9	0	32	15	35	2	25	2	21	2
46	56	23	29.1	13	2	16	0	7	0	18	0	7	0	20	0	27	0
52	225	25	10.0	4	2	10	0	6	0								
54	45	13	22.4	5	0	4	0										
62	80	0	0.0	37	2	40	1	39	0	44	0	39	1	18	0	0	0
63	46	18	28.1	0	0	24	2	13	0	8	0	17	0	12	0	0	0
64 C	27	13	32.5	Before change													
	58	28	32.5	After change													
66	110	58	34.5	26	20	11	20	16	13	27	2	16	0	9	0	0	0
Totals	2250	1191		279	115	414	205	257	81	368	44	245	9	376	4	273	5
Per cent. WD	34.6			29.2		33.1		23.9		10.6		3.54		1.05		1.79	

Controls were kept at 18 degrees Centigrade in continuous light for each group of experiments. When the experimental females were changed to the wing-reducing conditions, some of their sisters were placed upon a fresh plant, but allowed to remain in 18 degrees, light. The purpose of these controls was to show that wings did not decline for some reason other than the change of conditions. If indeed the latter were the case, the females continued at 18 degrees and in light might be expected to show a decline in wing production as well as their sisters which had been changed to the wing-reducing conditions. The results of these experiments are shown in Table 1.

It is of interest that the control females continued at 18 degrees Centigrade, light, produced more winged offspring in the latter part of the experiment than they did earlier. Thus these females produced 34.6 per cent. winged offspring before the experimental females were placed in the wing-reducing conditions, and 48.0 per cent. after their sisters had been transferred. The reasons for this increase in winged progeny is not apparent from the data; it shows, however, that while winged offspring born to the experimental females steadily became fewer in number, the percentage of winged offspring born to the control females increased in number.

The results shown in Table 1 may be summarized as follows:

Young born	WS	WD	% WD
At 18 degrees C., light	1423	638	30.95
At 20 degrees C., dark			
1st day	279	115	29.2
2nd day	414	205	33.1
3rd day	257	81	23.9
4th day	368	44	10.6
5th day	245	9	3.54
6th day	376	4	1.05
over 6 days	273	5	1.79
Controls, 18 degrees, light before change	535	283	34.6
Controls, 18 degrees, light after change	292	270	48.0
Total, 18 degrees C., light	2250	1191	34.6

From these data it may be seen that percentages of winged offspring born the first two days after the change of environmental conditions are approximately the same as

those born before the change. Aphids born on these days evidently were not affected by the suppressing factors in the environment. It may be assumed, therefore, that whatever determined whether an individual aphid was to be winged or wingless was settled sometime prior to two days before birth. If determination of wings were closer to birth than two days, say only a few hours before birth, then the only offspring expected to be winged would be those born within a few hours after the change of environmental conditions. Since, however, the aphids born for two days after the change of conditions showed approximately the same percentage of wings as those born before the change, determination sometime prior to two days before birth is certain. A percentage change may be noted in the third-, fourth-, fifth- and sixth-day offspring, with the minimal level always attained at 20 degrees in darkness, apparently reached after the sixth day.

Since there was a variation, in different experiments, in the number of days required before suppression of wings was complete, or nearly so, it was necessary to fix a mean time of determination of wings. The change from winged to wingless over a period of time can probably be best expressed by a regression line of the formula $y = a + bx$, in which the abscissae represent percentages of winged offspring and the ordinates represent successive days after the change from wing-producing to wing-inhibiting conditions. From the data on page 30, so far as they belong to the actual period of reduction of wings, this equation is computed to be $y = 5.12 - 0.112x$. The graph of this line and the points which it represents are shown in Fig. 1.

Females in 18 degrees Centigrade, continuous light, produced an average of 34.6 per cent. winged offspring, and the lowest level of wing production may be set at 1.5 per cent. without introducing a serious error. The difference between these two percentages, 33.1 per cent., would represent the extent of the change. The midpoint of this range, 16.5 per cent., representing 50 per cent. of the total change, could then be regarded as an objective measure of the mean time

needed for suppression of wings. In the formula, when x equals 16.5, the value of y is calculated to be $3.27 \pm .40$ days.

This figure, then, represents the average number of days between the time at which wing-influencing conditions were changed, and the time at which aphids were born which would have few or no wings. Since wings were determined

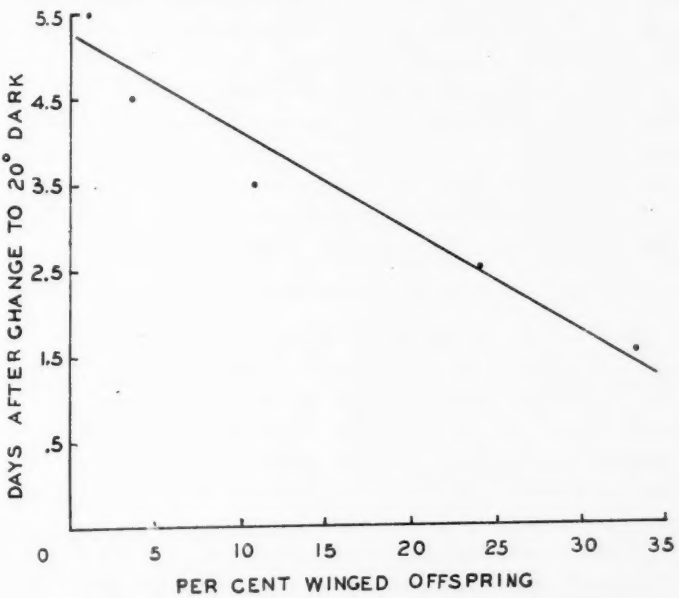


FIG. 1. Regression line for time of determination of wings.

by the wing-producing conditions, they must therefore have been determined, on the average, 3.27 days *before* the change of conditions. The average time of birth of aphids born during the first 24-hour period after the change of conditions is 12 hours, or 0.5 days, after the change. Since wings are determined 3.27 days before the change of conditions, they are determined 3.27 plus 0.5 or 3.77 days before birth. Determination of wing rudiments is therefore considered to have occurred at a mean time of about three and

three-quarters days before birth. It must be remembered that this is an average, and that in individual aphids the actual time of determination was sometimes before and sometimes after this time.

Another cause of the variation in the length of time needed for nearly complete disappearance of wings is probably irregular timing of individual births. If several embryos in different ovarioles are all at about the same stage in development, which ones are born first is probably a matter of chance. Thus a winged aphid might be born after a wingless one, even though its wings had been determined previously to the winglessness of the wingless aphid. Thus a variation in the number of days needed for suppression of wings might not indicate a difference in time of their determination, but only an accidental variation of a quantity used to calculate the mean time of determination.

Time of Differentiation of Wings

Shull (1928) has described the earliest traces of wings in *M. solanifolii.* The wings are first recognizable as thickenings of the hypodermis in the dorsolateral regions of the mesothoracic and metathoracic segments. The situation is somewhat complicated in that aphid, since fairly late embryos destined to be either winged or wingless possess hypodermal thickenings in the wing region. In wingless aphids no further development takes place and the thickenings eventually disappear, but in winged instars the rudiments grow thicker, evaginate, and finally form the wing pads characteristic of immature winged aphids. Considerable practice is needed to distinguish presumptive winged from wingless aphids on the basis of the wing thickenings in *M. solanifolii.* Thus the recognition of the time at which differentiation of these wing pads continues or is resumed in a direction destined to produce wings, is more difficult than in *M. sanborni,* as in the latter species no hypodermal thickenings are present at any stage in wingless forms. Shull (1938) found that the hypodermal thickenings in *M. solanifolii* were recognizable as developing wings, on the average, about 22–24 hours before birth.

White (1946), although not primarily concerned with the time of visible differentiation of wings, sectioned adults but found no evidence of hypodermal thickenings in unborn embryos. It was strongly suspected, therefore, that differentiation took place much later, perhaps after birth, and that thus a considerable time lag existed between determination and differentiation. See introduction, page 23, for further discussion of this point.

Wings are first visible externally in *M. sanborni* in the late second or early third instar, about four to five days after birth. Internal differentiation takes place then, sometime prior to the fourth or fifth day after birth.

It should be remembered that even the best wing-producing conditions resulted in families with only about 34 per cent. of the individuals winged. Therefore among all aphids in which wings could not be seen externally, only about one aphid in every three sectioned was expected to show internal evidences of wings.

The search for the delimitation of the time of wing differentiation was begun by making sections through the thoracic region of late second instar aphids, about four days old. These aphids possessed slight swellings upon the thorax, but it could not be told for certain from external observations that they were wing rudiments. Internally, however, the wings were plainly visible as thickened, heavily stained evaginations of the hypodermis.

Sections through three-, two-, and one-day-old aphids revealed smaller, but well-developed wing evaginations in about one-third of the individuals sectioned, and finally, in about a third of the aphids preserved within one hour after birth, definite recognizable thickenings in the proper regions of the thorax were evident. It was therefore concluded that wings must begin their differentiation before birth.

Accordingly, sections were made of adult wingless females which had been reared in 18 degrees Centigrade and continuous light. Similar hypodermal thickenings, in the same relative positions, were found in unborn embryos. Wings, then, are visibly differentiated before birth.

To ascertain the precise time of differentiation it was

necessary to have definite criteria of the hypodermal thickenings which were really wings. Younger instars, and especially unborn embryos, tend to have the hypodermis relatively thick and irregular at various places throughout the body. In order that thickenings be judged to be wings, therefore, certain requirements had to be fulfilled. The thickenings had to be in the correct body segments, that is, in the meso- and meta-thorax, and in the correct dorsolateral positions. Since each wing arises from a separate anlage the rudiments would be expected to appear at a certain section, grow thicker through a number of sections, then thinner, and disappear. The metathoracic wings would be expected to appear in the next few sections, thicken, and disappear. Thus early wing rudiments should be evident as paired thickenings of the hypodermis, one pair in the mesothorax, one pair in the metathorax, with a recognizably thinner portion of the hypodermis between them. Finally, embryos of the same age should have the thickenings in the same relative positions. The posterior border of the eye served as a satisfactory landmark. All sections were cut at ten micra. For each of the younger instars sectioned, records were kept of the following: (1) the distance behind the posterior border of the eye at which thickenings appeared, (2) the length (anteroposterior) of the mesothoracic disk, (3) the length of the interdisk thinning of the hypodermis, and (4) the length of the metathoracic disk when present. The mean values of these measurements are shown in Table 2 for unborn embryos, 0- to 1-hour instars,

TABLE 2

LOCATION OF WING RUDIMENTS IN IMMATURE APHIDS. ALL SECTIONS CUT AT TEN MICRA

Age in hours	Sections behind eye	Average length of mesothoracic disk (sections)	Interdisk thinning (sections)	Average length of metathoracic disk (sections)
Embryos	7.33	2.88	.....	.....
0-1	7.80	3.00	.....	.....
24	7.21	4.00	1.61	1.22
36	6.62	6.62	1.38	3.50

24-hour instars and 36-hour instars. Metathoracic rudiments do not appear until the 24-hour instars, so the table

for the earlier stages refers only to the mesothoracic disks. Thickenings which were properly placed, which continued through several sections, which were paired, and which were correctly spaced in relation to similar thickenings before or behind them, were regarded as rudiments of wings.

It then became necessary to determine the age of embryos with wing rudiments. Embryos of varying ages are found within each female, but the older ones may be recognized by their less compact appearance and lighter staining. Shull (1938) found that in *M. solanifolii* wing differentiation occurred at about the same time as did the pigmentation of the compound eye. The procedure outlined by Shull was checked for *M. sanborni* and it was found that again differentiation of wings closely followed the appearance of pigment in the ommatidia. As applied to *M. sanborni*, Shull's procedure is as follows: embryos with no pigmentation, or less than 50 per cent., showed no signs of wings; embryos with 50 per cent. or more pigmentation showed definite thickenings. Since only 34.6 per cent. of the aphids reared under these conditions were winged, only a similar per cent. could be expected in embryos with 50 per cent. or more pigmentation. The actual numbers obtained were somewhat higher, about 50 per cent., a variation (above 34 per cent.) due perhaps to errors of sampling, perhaps to higher numbers of wings resulting from some unknown factor.

From the facts, then, that embryos with eyes only slightly pigmented had no wing thickenings, while those with 50 per cent. pigmentation included at least as many winged ones as expected (actually more), it may be concluded that wing differentiation closely follows the first appearance of eye pigmentation.

The age at which pigmentation of the eyes takes place can be determined by a simple computation. Sections of fifty-five adult wingless females which had been reared at 18 degrees C., in continuous light, contained fifty-six embryos with eye pigment, or an average of 1.018 embryos per female. Under the same conditions, it had been determined that the mean number of young born per female per day

was 0.953. Therefore, the mean numbers of embryos with eye pigment, 1.018, divided by the mean number of offspring per female per day, 0.953, gives the number of days before birth that eye pigmentation appears. From these calculations, it appears that eye pigment is formed about 1.06 days before birth, and wing differentiation probably follows very shortly thereafter, or about twenty-four hours before birth.

Lag of Differentiation of Wings Behind Determination

It was found that wings are determined on an average of three and three-quarter days before birth. Differentiation takes place very close to one day before birth. The time lag of differentiation of wings behind their determination is thus approximately two and three-quarter days. Again it should be emphasized that these figures are averages and that individual aphids vary on either side of the mean. The time lag itself may be quite constant; the variation may be due to the different times of determination and subsequent differentiation in individual aphids. The probably variable ages of embryos at time of birth doubtlessly contribute to this apparent variation. See page 28.

Determination of Ocelli and Wing Muscles

Wingless adult aphids of this species lack ocelli and wing muscles, while winged forms possess them. It seems probable that the same process which determines wings also determines these structures. Whether the development of ocelli and wing muscles is due to an induction consequent upon the development of wings, or whether they are the result of pleiotropic effects of the wing determiner, is not known. Stiles (1938) concluded that in *M. solanifolii* the determination of the three structures is very close together in time, and if determination of ocelli and wing muscles did not take place simultaneously with wings, at least the determination of all three could not be separated. Since no winged aphid of the species *M. sanborni* was ever found to

lack ocelli and wing muscles, and none that had ocelli and wing muscles lacked wings, the assumption that ocelli and wing muscles are determined at the time of determination of wings, about three and three-quarter days before birth, probably introduces no serious error.

Time of Differentiation of Ocelli

Three ocelli are present in the winged adult. The median ocellus is in the center of the front, while the lateral ocelli are visible externally as rounded, lens-shaped differentiations of the cuticula, located dorsal to the eyes and slightly anterior to their centers. The ocelli are very small and difficult to see in mounted specimens; sections were made, therefore, of all specimens in which the presence of ocelli was in question. In section the ocelli are composed of two parts. Invagination of the hypodermis in the dorsolateral region of the head is often the first indication of the presence of ocelli. In older individuals these invaginations thicken and become connected with the dorsocentral portion of the brain. Ocellar rudiments do not grow out from the central nervous system. The second unit composing the ocellus is the lens, which appears after the internal portion of the ocellus is well formed.

Sections through the head region of winged nymphal instars revealed ocelli present in 100 per cent. of five- and four-day individuals. Aphids three days old showed 90 per cent. ocelli, and two-day nymphs 12.5 per cent. Aphids less than thirty-six hours old showed no evidence of ocelli. These results are shown in Table 3.

TABLE 3

Presence of Ocelli in Winged Instars of the Ages Indicated

Age in hours	With ocelli	Without ocelli	% with ocelli
0-1	0	22	0
13-36	0	46	0
37-60	1	7	12.5
61-84	9	1	90.0
85-108	36	0	100.0
109-132	13	0	100.0

The differentiation of ocelli takes place, on the average, after the second day and before the third day after birth.

Again applying the regression equation to the data, this equation is $y = 31.4 + 0.565x$.

The midpoint of the change from individuals without ocelli to those with ocelli probably best represents the average time of their differentiation. Hence by substituting 50 (per cent.) for x in the equation, we have $y = 31.4 + 0.565 \times 50$, or $y = 59.65$ hours. The graph of this line and the points which determine it are shown in Fig. 2.

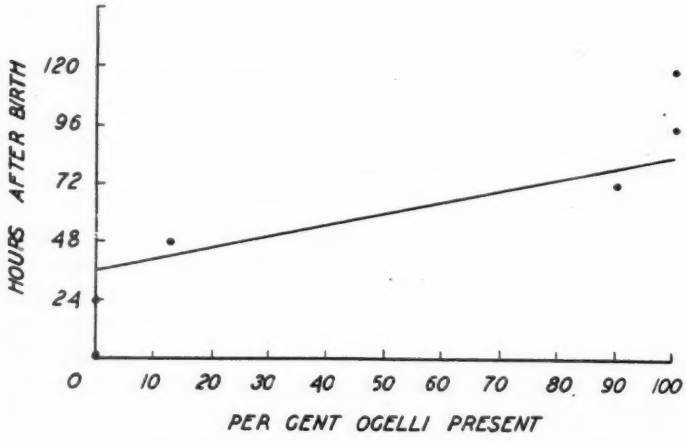


FIG. 2. Regression line for time of differentiation of ocelli.

Ocelli are differentiated, therefore, on the average, slightly less than two and one-half days after birth.

TIME OF DIFFERENTIATION OF WING MUSCLES

Closely associated with the possession of wings is the presence of wing muscles. It seems likely that they are determined by the same process which determines wings, for in older instars wings are never found without muscles and *vice versa*.

Sections through successively older embryos with thickened hypodermis might be expected to show the beginnings of differentiation of these muscles. Cross sections of winged aphids one hour or less old revealed no trace of wing

muscles in any individual. Winged aphids of an average age of 24 hours, however, showed muscles present in 6 of 42 individuals sectioned, or 14.2 per cent. Two-day aphids with hypodermal thickenings were half with, half without, wing muscles. All winged instars 72 or more hours old had muscles. The average time of differentiation takes place, then, between 24 and 72 hours after birth. Numbers of aphids of various ages possessing wing muscles are shown in Table 4.

TABLE 4

PRESENCE OF WING MUSCLES IN WINGED INSTARS OF THE AGES SHOWN

Age in hours	With muscles	Without muscles	% with muscles
0-1	0	22	0
13-36	6	36	14.2
37-60	8	8	50.0
61-84	17	0	100.0
85-108	48	0	100.0
109-132	14	0	100.0

On applying the regression formula to these data, so far as they belong to the period of change, the equation becomes:

$$y = 8.5 + 0.67x$$

The average time of differentiation of wing muscles as determined by the midpoint (50 per cent.) of the change from lack of wing muscles to complete possession of them, is represented by the equation:

$$y = 8.5 + 0.67 \times 50$$
$$y = 42.0$$

Differentiation of wing muscles takes place, therefore, 42 hours, on the average, after birth. The graph of this line and the points which determine it are shown in Fig. 3.

There seems to be a differential growth of the wing muscles in successive instars. The muscles in first-instar nymphs are small and uniformly pink-staining with eosin, and the nuclei are inconspicuous. In late second and early third instars, however, at about the time well-formed wing evaginations are evident, rapid changes take place in these muscles. The periphery of each muscle contains many dark-staining nuclei, while the interior remains pink. The

basophilic character of these peripheral nuclei indicates active mitoses. The change in the appearance of the muscles continues, so that in third-instar aphids, about five days after birth, the dark nuclei are present throughout the whole muscle. Active mitosis seems to stop at about the sixth day after birth, since large numbers of evident nuclei are not seen in muscle after this time. The muscles continue to grow in size, however, indicating that perhaps what happens is growth of the individual muscle cells.

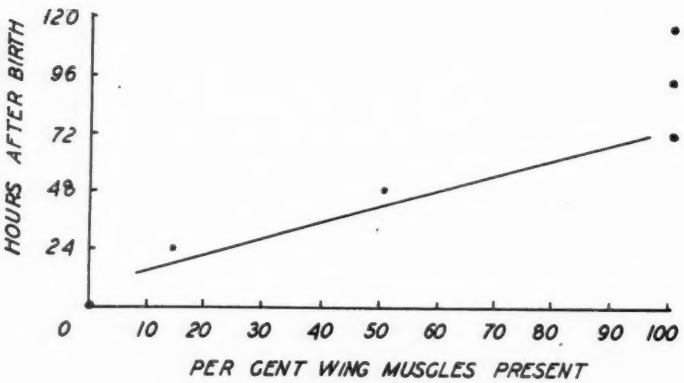


FIG. 3. Regression line for time of differentiation of wing muscles.

LAG OF DIFFERENTIATION OF OCELLI AND WING MUSCLES BEHIND THEIR DETERMINATION

If the time of determination of ocelli and wing muscles is contemporaneous with the time of wing determination, the lag of differentiation of these structures behind their determination is considerably greater than that of the wings themselves. The significance of this greater lag is not clear-cut, however, as the exact time of determination of the ocelli and wing muscles is somewhat in doubt. The order of differentiation of these structures in *Macrosiphum sanborni* is clearly wings first, then wing muscles, then ocelli. The order of their determination is not known.

Discussion

Of the various agencies which have been proposed to account for the production of wings in aphids, the one suggested by White (1946) seems to fit the data for *M. sanborni* better than any other. Adapting the scheme suggested by Shull (1929a) to her results, White suggested that wing production could result from some substance A, produced in the light but not in the dark, or perhaps changed into some other substance B in the dark. Low temperatures result in a greater quantity of A, either by an acceleration of production or a retardation of its conversion, or perhaps both. In another aphid, Ackerman (1926) concludes that wing production is " dependent upon changes in the proportion or concentration of certain materials in the haemolymph as brought to pass by the rupture of the brown globules." It is probable that what Ackerman regarded as haemolymph was in reality the fluid content of the large vacuolated cells which fill the body cavity of aphids. These are the " adipose cells " of Uichanco and Weber, and when Ackerman crushed his aphids the fluid streaming from these cells could be easily taken for haemolymph. Nevertheless, the important consideration for the present purpose remains the same; a presumably diffusible substance is produced under certain conditions, not produced under others, which ultimately results in wing production. Shull (1943) concludes that whatever the mechanism by which wings are produced, experimental results indicate that heat and light act through the same agent, supposedly some substance produced in the body of the aphid. Other investigators have found that starvation, crowding on the plant, water balance in the insect's body, relative humidities, type of parent, number of generations of winglessness, and numerous other factors contribute to the presence or absence of wings. Presumably all these environmental effects must ultimately work through a diffusible substance in the aphid's body which affects in some way or other the cells

which will ultimately give rise to wings and associated structures.

If indeed such is the case, differences in the action of this substance, whatever it may be, should begin to afford some clues as to its nature. As stated above, Shull (1938) found that in the potato aphid the time lag between determination and differentiation of wings was approximately 12 hours. In the chrysanthemum aphid this period is now found to be about two and three-quarter days.

Such a difference in time of action of this "substance" might be explained in at least two ways. If the substance produced were the same in both species of aphid, a difference in rapidity of action might be explained by a genetic difference in the cells of the two species, which reacted positively or negatively to the influence of the diffusible substance. If on the contrary the genetic factor were minimized (although a difference as small as one gene could easily cause the results obtained), then many differences in wing production might be explained by the hypothesis that a difference existed in the kind of substance produced by the two aphids. Perhaps both of these suppositions are true to a certain extent.

The second assumption, namely, that each aphid produces a different kind of diffusible substance, might explain several of the differences between the species. In the potato aphid continuous light or darkness inhibits wing production, but an alternation of light and darkness promotes it. In the chrysanthemum aphid, however, continuous light is the most important wing-producing factor. This distinction between the two species is somewhat reduced by the fact that at least some strains of *M. solanifolii* do not show the typical response to light. Shull (1932) demonstrated that a laboratory strain of the potato aphid suddenly reversed its response to light so that continuous light promoted rather than restricted wing production. It was also demonstrated by Shull (1943) that some strains collected from nature exhibited this opposite response to light. Some other strains investigated showed an intermediate response

to light, as measured by amount of wing production. The characteristic behavior of the species, nevertheless, are almost opposite with respect to response to conditions of light.

Temperature effects were approximately the same in both species, high temperatures retarding wings and low temperatures stimulating or at least not inhibiting them. In the potato aphid it seems necessary to postulate two substances, A and B, one of which is produced in the light, the other in the dark. Wings are produced when A and B are in a certain ratio of abundance, or when a minimal quantity of B is present. It seems from the data for the chrysanthemum aphid (White, 1946) that this ratio is not the important thing, but rather the production of some substance which we might call A′, favored by continuous light and low temperatures. A difference in the chemical nature of these substances in the two species of aphids could conceivably explain the differences in their response to light and temperature.

Another possibility not precluded by the data is Goldschmidt's hypothesis that structures such as wings develop as the response of a rudiment to a chemical evocator, produced at the right time in the development of the individual. Wings, for example, would be due to a combination of two factors: (1) the presence, presumably genetic, of a wing rudiment, which, given proper conditions, could become a wing, and (2) the presence of a chemical evocator produced somewhere in the embryo, which would affect the disk (rudiment) during a certain critical period. Thus wing production would depend on the release of the evocator during a certain critical period during which the disk is capable of responding to it. In *M. sanborni* the data might be fitted to this theory as follows: Higher temperatures are assumed to affect the maturation of the disk (and the insect in general) by increasing the rate of development. It possibly also has some effect upon the rate of production of the evocator. Light is presumed to affect the production of the evocator more than it does the rate of embryonic develop-

ment. Thus in continuous light, at lower temperatures, the maturation of the cells destined to become wings is slowed down while at the same time the production of the evocator is accelerated, and as a result the evocator is released at the same time that the disk becomes mature, and wings result. This assumes that under ordinary conditions of light and temperature, the wing rudiment becomes mature (capable of being acted upon by the evocator) considerably before the evocator is released. Under room conditions, for example, a higher temperature speeds up development of the disk, while the partial darkness slows down production of the evocator, so that the disk is already "determined" in the direction of winglessness by the time that a sufficient amount of the evocator is present, if indeed it ever is present in the absence of continuous light. High temperatures, even in continuous light, would speed development of the disk faster than the production of the evocator, hence lesser numbers of wings. Low temperatures and partial darkness again produce no wings, presumably now from the lack of sufficient production of the evocator. All these assumptions are based upon the data given by White (1946).

One feature seems to be consistent. The evocator, organizer or diffusible substance appears in all cases to be a chemical substance produced somewhere in the body, and capable of influencing the development of certain cells. Once this development has been initiated it is possible that the developing wing tissues produce substances which influence, by induction, the development of ocelli, or perhaps the ocelli result from later effects of the same substance which initiates wing development. In the latter case, following Goldschmidt, the ocelli rudiments would be "mature" considerably later than the wing rudiments.

The relatively longer period between determination and visible differentiation in this species suggests the possibility of an inductive influence. If the proper conditions of light and temperature produced a "determining" effect in some tissue, perhaps the diffusion of a product of that stimulation, through cells and tissues, gives the stimulus for the

thoracic hypodermis to thicken. This induction would presumably be a slower process than the effect of a chemical substance liberated, say, in the body fluid. The longer time necessary for differentiation of the ocelli and wing muscles might also be explained by appeal to such an induction.

Are there any data as to the locus of the production of this diffusible substance? Goldschmidt refers to it as being produced by the "germ," although in no specific location. Shull (1929a) wonders whether the substance is produced in the embryo or in the mother. Again merely on theoretical grounds, is it possible that an enzyme able to affect structural characters is produced in the mycetom of aphids? According to Buchner (1930) the mycetom is composed of symbiotic yeasts. It is not difficult to imagine the necessary enzyme production by yeasts in the body cavity. Macroscopically the mycetom appears green or brown.

If indeed the mycetom is composed of symbiotic yeasts, light and temperature would conceivably have a considerable effect upon enzyme production. Mycetom constituents are known to be passed from mother to embryo in the aphids; perhaps a quantitative or qualitative difference in the kinds of yeasts present would account for the differences in behavior of aphids under the same environmental conditions. Mutations might well be, as Shull (1943) suggests, a function of the mycetom. At least three different types of symbionts are known to occur in aphids and a comparative study of the mycetom might yield substantial results. Further work with the mycetom and symbionts seems definitely to be in order.

The relatively long period of time between determination and differentiation of wings in this species suggests at least two possible alternatives to account for the ultimate production of wings. One of these alternatives postulates a lengthy, or at least time-consuming, chemical reaction, or more probably a chain of such reactions. The chain is either initiated by the proper environmental conditions, or some blockade is removed which permits the chain to be carried to its completion.

The other explanation is based upon a series of inductive changes. A change begun by the proper environmental conditions initiates a stimulus which is carried from cell to cell and from tissue to tissue, ultimately reaching and affecting the hypodermal cells which give rise to wings. Perhaps this latter explanation is only a special case of the former—inductive changes are presumably of a chemical nature.

If indeed a series of chemical changes is involved, perhaps certain chemicals may be found which will produce the same effects as the wing-stimulating environmental conditions. If this can be done, the process can be analyzed more fully and the intermediate steps discovered.

Summary

1. Approximately 10,000 chrysanthemum aphids (*Macrosiphum sanborni*) were raised in an investigation of the relationship of the time of determination of wings and related structures to the time of their visible differentiation.

2. Determination of wings takes place, on the average, three and three-quarter days before birth.

3. Differentiation of wings takes place approximately one day before birth, shortly after the initial pigmentation appears in the compound eyes.

4. About two and three-quarter days, therefore, elapse between determination and differentiation of wings.

5. Since ocelli and wing muscles are found only in winged forms, the determination of these structures is assumed to be fixed at the same time that wings are determined.

6. The average time of differentiation of ocelli is about two and one-half days after birth, and of wing muscles about one and three-quarter days after birth. If the assumption that these structures are determined at the same time as wings is correct, then a considerably longer time elapses between their determination and visible differentiation.

7. The action of a diffusible chemical substance is suggested as a possible interpretation of the relatively long

time period between determination and differentiation of wings.

LITERATURE CITED

Ackerman, Lloyd
1926. *Jour. Exp. Zool.*, 44: 1–61.
Buchner, P.
1930. "Tier und Pflanze in Symbiose." Borntraeger. Berlin. 1–900.
Dawydoff, C.
1928. "Traité d'embryologie comparée des invèrtébrés." Mass. et Cie. Paris. xiv + 930.
Gillette, C. P.
1908. *Canad. Ent.*, 40: 61–68.
Goldschmidt, Richard
1938. "Physiological Genetics." McGraw-Hill Book Co. ix + 375.
1940. "The Material Basis of Evolution." Yale University Press. ix + 436.
del Guercio, G.
1911. *Redia*, 7: 296–333.
Hottes, F. C., and T. H. Frison
1931. *Bull. Nat. Hist. Surv. Ill.*, 19: 121–447.
Johannsen, O. A., and F. H. Butt
1941. "Embryology of Insects and Myriapods." McGraw-Hill Book Co., ix + 462.
Kitzmiller, J. B.
1948. *Trans. Am. Micro. Soc.*, LXVII: 227–230.
Marshall, W. S.
1935. *Jour. Morph.*, 57: 571–596.
Patch, E. M.
1938. *Bull. Maine Agr. Exp. Sta.*, 393: 35–431.
Patten, W.
1887. *Jour. Morph.*, 1: 193–226.
1888a. *Jour. Morph.*, 2: 97–190.
1888b. *Jour. Morph.*, 2: 600–602.
Sanborn, C. E.
1904. *Kansas Univ. Sci. Bull.*, 3: 1–82.
Shull, A. F.
1928. *W. Roux' Arch. Entw. d. Org.*, 113: 210–239.
1929a. *W. Roux' Arch. Entw. d. Org.*, 115: 825–851.
1929b. *Biol. Rev.*, 4: 218–248.
1930. *Zeitsch. ind. Abst. Vereb.*, 57: 92–111.
1932. Am. Nat., 66: 385–419.
1933. *Proc. Nat. Acad. Sci.*, 19: 164–175.
1935. *Biol. Bull.*, 68: 35–50.
1937. *Biol. Bull.*, 72: 259–286.
1938. Am. Nat., 72: 170–179.
1942. *Jour. Exp. Zool.*, 89: 183–195.
1943. *Papers Mich. Acad. Sci., Arts and Letters*, 28: 425–431.

Snodgrass, R. E.
1935. "Principles of Insect Morphology." McGraw-Hill Book Co. ix + 401.
Spemann, Hans
1938. "Embryonic Development and Induction." Yale University Press. xii + 401.
Stiles, Karl A.
1938. *Biol. Bull.*, 74: 430–460.
Theobald, F. V.
1926. "The Plant Lice, or Aphididae, of Great Britain." I. Headley Brothers, London. ix + 372.
Uichanco, L. B.
1924. *Philip. Jour. Sci.*, 24: 143–247.
Weber, H.
1930. "Biologie der Hemipteren." Julius Springer. Berlin. vii + 543.
White, W. S.
1946. Am. Nat., 80: 245–270.

THE RELATIONSHIP BETWEEN TEMPERATURE, RATE OF DEVELOPMENT AND EXPRESSION OF THE PUFDI GENE IN *DROSOPHILA MELANOGASTER*[1]

PERCY H. BAKER

VIRGINIA STATE COLLEGE

STUDIES of non-autonomous genes in *Drosophila melanogaster* have disclosed that (1) in most cases, the degree to which the gene is expressed depends upon the temperature at which the flies are reared, and (2) temperature is effective for only a limited part of the embryonic life of the fly generally referred to as the temperature-effective period. One of the explanations proposed for the effect of temperature on gene expression is that temperature produces a differential change in either the rate or duration of the embryonic processes leading to the production of the character in question as compared with the rate of total embryonic development (Seyster, 1919; Zeleny, 1923; Driver, 1926; Stanley, 1931; Riedel, 1934; Harnly, 1936). The mutant *Pufdi* exhibits some features which suggest that it might yield additional information on the relationship between temperature, rate of development and gene expression. The data which follow are the results of studies to determine these relationships.

THE GENETIC NATURE OF PUFDI

The mutant *Pufdi* originated in a wild stock in the genetics laboratory at the University of Michigan and was first described by Shull in 1938. The name is a contraction of the words "puffed" and "divergent" which are descriptive of the wings. These are occasionally puffy due

[1] Contribution from the Department of Zoology at the University of Michigan.

to the inclusion of a bubble of fluid between their membranes, and they diverge laterally from their points of attachment to the thorax so that in typical cases, their outer margins describe a considerable angle. Shull noted that the angle formed by the wings was greater in cultures reared at high temperatures. In these studies the spread of the wings was used as the measure of gene expression.

The Pufdi gene is dominant and lethal when homozygous. Shull determined its locus to be 70.8 on the second chromosome. Its proximity to Lobe^2, another dominant, homozygously lethal, second-chromosome gene (locus 72.0) permits these two to be carried in combination as a balanced lethal stock (Pfd/L^2). In addition to simplifying the maintenance of stocks, this arrangement also facilitates recognition of Pufdi flies. Wild females mated to Pfd/L^2 males yield Pfd/+ and L^2/+ progeny in a 1:1 ratio. All non-lobe flies are regarded as Pufdi irrespective of wing spread.

Experimental Procedure

Experimental cultures were started with eggs which had been secured by lowering, by means of wire loops, bottle caps of yeasted food into half-pint bottles containing from 25 to 30 pairs of parents. The food caps were removed after two-hour egg-laying intervals and the eggs distributed among standard yeasted food bottles at the rate of 100 eggs per bottle.

Wing spread is expressed in terms of degrees of the angle formed by the relatively straight parts of the outer margins of the wings. A protractor of transparent material was mounted on a wood base with shims providing an intervening space which accommodated a radius bar. This bar was pivoted at the center of the base line of the protractor and projected beyond the margin of the protractor so that it could be rotated to describe, with the base line, any angle up to 180°. Etherized flies were placed on their dorsal surfaces on a glass slide and this manipulated over the surface of the protractor until the outer margin of one wing coincided with the base line of the protractor. Then the radius

bar was adjusted until it coincided with the outer margin of the outer wing and the angle read off to the nearest degree. Measurements were made after the wings of the newly emerged adults had fully matured as evidenced by their dry and more or less brittle texture and by the development of normal pigment in the body. Measurements were made twice daily, approximately twelve hours apart, during the time of emergence. Any very young imagoes taken at one period were returned to the culture bottle to be measured at the next period.

The Effect of Temperature on Wing Spread

Eggs collected from $+/+ \times \text{Pfd}/\text{L}^2$ parents and from wild type (Oregon-R♀) controls were incubated at 16°, 19°, 22°, 25°, 28° and 31° C. It was originally intended to keep the parents and collect eggs at each experimental temperature so that the entire development, including formative stages of the egg, would be subject to the influence of these temperatures. This was found to be impractical, however, due to the slow rate of oviposition at low temperatures. As a compromise, matings were maintained and eggs collected at 25° C., and the eggs immediately transferred to the cultures at which it was desired to rear them. The culture bottles to receive the eggs were put at the desired temperatures several hours before the eggs were placed in them and returned immediately afterwards to the same temperatures. Thus, except for the initial 0–2 hours of the egg stage, development was entirely at the experimental temperatures. Later developments in the course of the experiments have indicated that temperature at early embryonic stages is without effect on wing spread, so the precautions observed seem adequate.

The wing spread of Pfd/+ progeny and wild-type controls was measured for each temperature. The variation in wild flies was so slight that it is justifiable to conclude that temperature has no effect on the wing spread of wild flies. The data on Pfd/+ are given in Table I. It is apparent that the wing spread of both male and female Pfd/+ flies

TABLE I

THE WING SPREAD OF PUFDI FLIES AT DIFFERENT TEMPERATURES

Temp. °C.	M:Males	M:Females
16	36.289 ± 2.485	27.355 ± 1.870
19	31.272 ± 1.326	25.881 ± 1.068
22	59.800 ± 3.328	40.022 ± 2.324
25	74.392 ± 2.603	66.304 ± 2.060
28	95.993 ± 2.216	87.125 ± 1.859
31	115.508 ± 1.884	104.777 ± 1.700

increases with each 3° increase in temperature between 19° and 31°. Below 19°, the inhibitory effect of low temperatures does not continue progressively. On the contrary, wing spread at 16° exceeds slightly, but perhaps not significantly, that at 19°.

The data are summarized graphically in Fig. 1, which emphasizes (1) the sharp, almost rectilinear increase in the

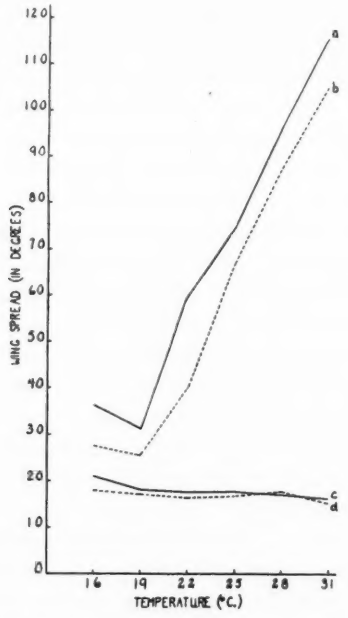


FIG. 1. Temperature and wing spread.

a—Pfd/+ males. c—+/+ males.
b—Pfd/+ females. d—+/+ females.

wing spread of Pfd/+ flies beginning at 19°, (2) the negligible change of the wild type, and (3) with the exception of the 28° wild population, the consistently greater wing spread of males.

The Temperature-Effective Period

Pufdi flies reared at constant temperatures for their entire embryonic life exhibited maximum and minimum wing spreads at 31° and 19° respectively. Transfers between these temperatures, therefore, were employed to determine the period in the life history at which a change in tempera-

TABLE II

Wing Spread of Pufdi Flies—Development Begun at 19° and Completed at 31°

Days at 19°	M:Males	M:Females
1	108.448 ± 3.031	94.512 ± 3.342
2	121.279 ± 2.059	100.714 ± 3.549
3	122.308 ± 3.680	111.875 ± 3.270
4	106.818 ± 3.698	96.548 ± 3.084
5	110.930 ± 1.623	98.221 ± 1.950
6	116.897 ± 3.155	108.333 ± 3.925
7	117.061 ± 2.162	108.125 ± 2.745
8	110.500 ± 2.905	115.555 ± 2.002
9	109.737 ± 4.277	110.610 ± 3.445
10	101.431 ± 2.199	95.167 ± 2.623
11	117.727 ± 2.625	102.747 ± 4.424
12	106.837 ± 3.012	101.795 ± 3.527
13	117.655 ± 2.172	105.909 ± 2.606
14	110.150 ± 2.074	104.504 ± 2.287

Wing spread when entire development is at 19°—
Males: 31.272 ± 1.326
Females: 25.881 ± 1.068

Wing spread when entire development is at 31°—
Males: 115.508 ± 1.884
Females: 104.777 ± 1.700

ture would produce a modification of wing spread. Cultures were prepared as in the previous experiments and started at 19°. Beginning after 24 hours and for each successive 24-hour interval thereafter up to 14 days (adults began to emerge by the 15th day), cultures were transferred to 31° to complete their development. A series of reciprocal experiments was arranged in which cultures were started at 31° and transferred at consecutive 24-hour intervals to 19°. At 31°, the cultures usually had some newly emerged adults by the seventh day, so transfers were made through 6 days. The results of these two series of experiments are given in Tables II and III.

In all cases, the wing spread of flies developing in the transferred cultures approximated that which is characteristic of the temperature at which the flies complete their development regardless of how long they were exposed to the initial temperature. It is necessary to account for the occasional statistically significant deviations of the transferred populations from these values. Sources of error which can be readily recognized include (1) reliability of the measurements of wing spread, (2) the constancy of the temperature cabinets used, and (3) the variety of conditions which might develop in different culture bottles. These possible sources of error are discussed in a later section. It may be stated here, however, that they appear to

TABLE III

WING SPREAD OF PUFDI FLIES—DEVELOPMENT BEGUN AT 31° AND COMPLETED AT 19°

Days at 31°	M: Males	M: Females
1	23.703 ± 0.953	21.923 ± 0.888
2	28.613 ± 1.467	25.461 ± 2.051
3	47.564 ± 2.582	35.992 ± 2.080
4	33.688 ± 2.096	21.166 ± 0.913
5	41.163 ± 2.508	29.182 ± 1.632
6	33.685 ± 1.523	31.210 ± 1.122
Wing spread when entire development is at 19°—		
	Males: 31.272 ± 1.326	
	Females: 25.881 ± 1.068	
Wing spread when entire development is at 31°—		
	Males: 115.508 ± 1.884	
	Females: 104.777 ± 1.700	

be ample to account for the variations in wing spread which appear in Tables II and III. It is fairly certain that flies which are transferred from 19° to 31° at any time between the first and fourteenth day of their embryonic life will exhibit a wing spread which is not different from that characteristic of 31° cultures. Thus the temperature-effective period at 19° appears to lie between the fourteenth day of development and eclosion, and at 31° between the sixth day of development and eclosion. In both instances, this is within the last 24 hours of the pupal period.

To determine more closely the limits of the temperature-effective period, a series of experiments was arranged in which the cultures were started at 19° and kept at that temperature until adults began to emerge. These adults

were discarded, the bottles warmed quickly by holding them for a few minutes in water at 34°–35° and then placed in a cabinet at 31°. Adults which emerged subsequently were collected at two-hour intervals, held at 31° until the wings were mature and then measured for wing spread. In this way, wing spread was determined for a series of flies that had been transferred from 19° to 31° between 0 and 2, 2 and 4, 4 and 6, 6 and 8, 8 and 10 and 10 and 12 hours prior to emergence. The data from these experiments are given in Table IV.

TABLE IV

WING SPREAD OF FLIES TRANSFERRED FROM 19° TO 31° IMMEDIATELY PRIOR TO EMERGENCE

Hours at 31°	M:Males	M:Females
0- 2	89.2850 ± 3.119	87.121 ± 2.682
2- 4	77.013 ± 4.546	82.458 ± 3.980
4- 6	92.826 ± 4.215	106.725 ± 3.618
6- 8	110.500 ± 4.132	107.813 ± 3.668
8-10	106.785 ± 3.902	113.055 ± 5.870
10-12	110.440 ± 3.977	104.115 ± 4.805

In interpreting the response of these pre-emergence transfers, it is necessary to calculate the beginning of the temperature-effective period from the time of emergence. Males which have emerged after 6 to 8 hours at 31° are completely changed to the characteristic for that temperature. Periods beginning later than 6 to 8 hours before emergence result in intermediate degrees of wing spread. A period beginning 6 to 8 hours before emergence is therefore the latest one at which a change from 19° to 31° will result in a complete transition of the wing character. Presumably then, these flies have been at the 31° temperature for the duration of the effective period. The lesser wing spread of flies emerging after shorter intervals may be charged to exposure to a 31° temperature for less than the total effective period and so they were only partially modified. Those which emerged within two hours after being transferred differ significantly from the 19° characteristic, which means that temperature operates during this interval also. The possibility that temperature continues to be effective after emergence is nullified by the fact that flies

emerging at all intervals were kept at 31° until the wings matured without apparent effect on the spread of the wings. The evidence is therefore that at 19°, the temperature-effective period in males begins between 6 and 8 hours before emergence and continues up to the emergence of the adult from the puparium. In females at the same temperature, the period begins between 4 and 6 hours before emergence and continues up to that event.

A similar series of pre-emergence transfers from 31° to 19° was arranged. Adults were again collected at two-hour intervals as they emerged and were held, in this case at 19°, until their wings hardened. The data from these transfers are given in Table V.

TABLE V

WING SPREAD OF FLIES TRANSFERRED FROM 31° TO 19° IMMEDIATELY PRIOR TO EMERGENCE

Hours at 19°	M: Males	M: Females
0- 2	111.014 ± 4.722	110.862 ± 3.445
2- 4	87.357 ± 5.641	88.533 ± 4.684
4- 6	70.735 ± 6.212	86.087 ± 5.449
6- 8	50.536 ± 4.828	46.520 ± 4.251
8-10	41.528 ± 5.464	29.224 ± 3.969
10-12	32.344 ± 3.127	27.916 ± 3.494

Employing the same interpretation that was used in the 19° to 31° transfers, the latest period in the embryonic life of the fly at which a change from 31° to 19° will effect a complete transition in the character of the wing is one which begins between 8 and 10 hours before emergence in both males and females. The effective period does not extend up to emergence in this case, but ends between 2 and 4 hours prior to it.

The results of these two groups of pre-emergence transfers are summarized graphically in Fig. 2. In plotting the length of the exposure to the second temperature (abscissa) the midpoints of the two-hour intervals at which flies emerged are employed. The lines show the rapid transition which the change in temperature causes in the spread of the wings, and the period (temperature-effective period) during which the wing is susceptible to modification by temperature.

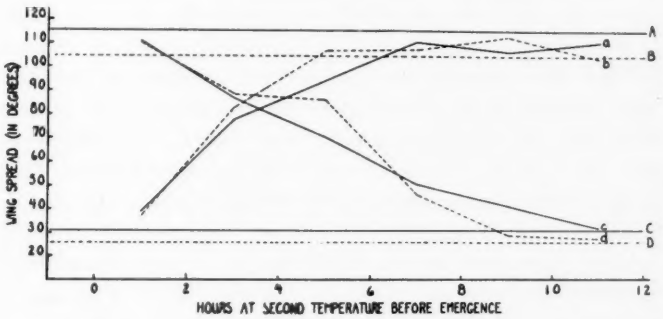


FIG. 2. Wing spread of Pfd/+ flies transferred between 19° and 31° immediately prior to emergence.

a—males transferred from 19° to 31°. c—males transferred from 31° to 19°.
b—females transferred from 19° to 31°. d—females transferred from 31° to 19°.
(A, B, C, D—wing spread of flies which underwent entire development at one temperature: A—males at 31°, B—females at 31°, C—males at 19°, D—females at 19°.)

THE EFFECT OF OTHER GENES ON THE WING SPREAD OF PUFDI

Combinations of Pufdi with *Lobe*^{2}, *Minute* (*3*) *w*, and *garnet*^{2} were made to give the series of genotypes listed in the first column of Table VI. The reasons for the choices

TABLE VI

WING SPREAD OF PUFDI-AND-MUTANT COMBINATIONS, (AT 25° C.)

Genotype	M:Males	M:Females
Pfd/+	74.392 ± 2.603	66.804 ± 2.060
Pfd/L^2	59.346 ± 2.956	65.475 ± 2.743
Pfd L^2/++	81.423 ± 3.860	82.445 ± 3.720
Pfd/+, M(3)w/+	78.425 ± 3.586	65.578 ± 3.656
gt^2/O, Pfd/+	86.306 ± 2.605	
gt^2/gt^2, Pfd/+		80.633 ± 2.310

of mutants were several. The Pufdi stock is, as a matter of convenience, carried in combination with the Lobe^2 gene in the arrangement: Pfd/L^2. For this reason, it was desired to know if the presence of the L^2 gene had any effect on the expression of Pufdi. In addition, the possibility of the arrangement Pfd L^2/++ provided an opportunity to test for a possible position effect on the L^2 gene. The other

genes were selected because they are known to modify rate of development or to affect the expression of other variable genes. The M(3)w gene prolongs development (Brehme, 1941) and influences the expression of Dichaete[4] (Plunkett, 1926) and Lobe[2] (Dunn and Coyne, 1935). Garnet[2] modifies considerably the facet number in Bar eye (Hersh, 1929).

The experimental cultures were again made up of eggs 0 to 2 hours old, distributed at the rate of 100 eggs per bottle. This time, all the cultures were incubated at 25° C. Data on wing spread are given in Table VI.

The garnet[2]-Pufdi line has greater wing spread than Pufdi in both sexes. Lobe[2] in the combination Pfd/L^2 seems to reduce wing spread in males, and in the combination Pfd L^2/++ it increases it in females. The other Pufdi-and-mutant combinations are probably not different from Pfd/+.

The Lobe[2] gene exhibits two noteworthy effects. When the two arrangements with Pufdi are compared, there is evidence that the position of the Lobe[2] gene does affect wing spread. In both males and females, the wing spread of the Pfd L^2/++ arrangement exceeds that of the Pfd/L^2 arrangement. Thus:

	M: Pfd L^2/++	M: Pfd/L^2
Males	81.423 ± 3.860	59.346 ± 2.956
Females	82.445 ± 3.720	65.474 ± 2.743

The other peculiarity of the Lobe[2] gene is that in both combinations with Pufdi, it cancels the sexual dimorphism which is otherwise characteristic of wing spreads. This becomes apparent when males and females possessing the Lobe[2] gene are compared as follows:

Genotype	M: Males	M: Females
Pfd/L^2	59.346 ± 2.956	65.475 ± 2.743
Pfd L^2/++	81.243 ± 3.860	82.445 ± 3.720

The non-Lobe[2] lines exhibit a distinct sexual dimorphism with males having the greater wing spread.

The Effect of Temperature on Rate of Development

In the absence of techniques which would permit the velocity of a particular developmental process to be measured directly, possibly the closest approach is to regard the rate of total development (*i.e.*, the time required for the entire embryonic development) as the sum of the rates of component individual processes and then look to find differences in the rates of individual processes reflected as changes in the total rate. From this point of view, if wild and Pufdi flies are reared together at low temperatures where they are not very different phenotypically, they might be expected to exhibit more nearly the same rate of development than if they were reared at high temperatures where they are phenotypically most unlike. There is, of course, the alternate and certainly the equally likely possibility that changes in the rate of an individual process have no effect on the duration of the total embryonic period. Failure to find a relationship, therefore, between the total rate of development and gene expression would not negate the rate hypothesis. However, the presence of such a relationship would be regarded as evidence supporting this hypothesis.

Accordingly, the rate of development of Pufdi flies and their wild-type controls was determined for each experimental temperature. Beginning with the appearance of the first adult, these were removed at two-hour intervals until no more appeared in the bottle. The duration of development was calculated as extending from the midpoint of the two-hour egg-laying interval to the midpoint of the two-hour period in which the adults emerged. The fact that the first two hours of the egg stage were spent at 25° introduces a slight error in the values except for the rate at 25°. However, since the practice was followed for both Pufdi and wild cultures, the values obtained are valid for comparison of these two lines.

Results of the experiments are given in Table VII. With the exception of males at 28°, the rate of development of

TABLE VII

DURATION OF DEVELOPMENT (IN HOURS) OF PUFDI AND WILD FLIES

Temp. °C.	Males		Females	
	Pufdi	Wild	Pufdi	Wild
16	617.136 ± 1.008	627.184 ± 0.804	597.553 ± 0.897	612.728 ± 0.816
19	371.149 ± 0.765	377.685 ± 0.725	361.322 ± 0.849	366.449 ± 0.966
22	267.372 ± 0.352	268.994 ± 0.335	265.388 ± 0.378	268.409 ± 0.368
25	208.301 ± 0.353	212.744 ± 0.355	201.009 ± 0.295	209.116 ± 0.375
28	184.067 ± 0.367	184.234 ± 0.411	179.150 ± 0.331	181.274 ± 0.415
31	176.808 ± 0.299	178.442 ± 0.417	172.948 ± 0.333	177.702 ± 0.441

Pufdi and wild flies differ meaningfully at all temperatures. The difference is not less at low temperatures where the two lines are phenotypically most alike. This would be at 19°. Actually, the difference is greater at this temperature than at some of the higher temperatures marked by a greater divergence in phenotype. There seems to be no evidence that differences in the expression of the Pufdi gene are associated with differences in rate of development.

THE EFFECT OF OTHER GENES ON RATE OF DEVELOPMENT

If gene expression is a consequence of developmental rate, the Pufdi-and-mutant combinations which exhibit greatest wing spread (Table VI) should also exhibit the most rapid rates of development. Thus a comparison of rates of development of Pufdi and Pufdi-and-mutant combinations provides another test, in terms of total development, of gene expression.

Rate of development at 25° C. was determined for each line for which wing spread had been determined. Two groups of experiments were conducted. The first group utilized *Pfd*/+, *Pfd L*^{2}/++, and *Pfd*/+, *M*(3)*w*/+ stocks and the second group *Pfd*/+, *Pfd*/*L*^{2}, *Pfd*/+, *M*(3)*w*/+, *gt*^{2}/*O*, *Pfd*/+, and *gt*^{2}/*gt*^{2}, *Pfd*/+ stocks. In each group of experiments, cultures of all the stocks used were started at approximately the same time, were reared together in the same temperature cabinet, were rotated about the shelves to minimize temperature differences within the cabinet, and finally the adults were collected at the same time intervals. The results are given in Table VIII.

TABLE VIII

DURATION OF DEVELOPMENT OF PUFDI AND PUFDI-AND-MUTANT STOCKS (IN HOURS, AT 25° C.)

Genotype	M:Males	M:Females
Exp. 1: Pfd/+	211.114 ± 0.392	203.642 ± 0.494
Pfd L^2/++	212.798 ± 0.404	210.180 ± 0.461
Pfd/+, M(3)w/+	277.474 ± 1.234	273.044 ± 1.403
Exp. 2: Pfd/+	218.802 ± 0.641	213.096 ± 0.590
Pfd/L^2	209.164 ± 0.511	208.572 ± 0.507
Pfd/+, M(3)w/+	289.084 ± 1.826	283.852 ± 1.940
gt^2/O, Pfd/+	222.994 ± 0.540	
gt^2/gt^2, Pfd/+		214.619 ± 0.470

Before comparing the combinations with Pfd/+, it should be noted that the rate for the Pfd/+ stock itself differs in the two groups of experiments. The *Pfd/+, M(3)w/+* stock was also used in both groups of experiments and the fact that it also required several hours longer to develop in the second group strongly suggests a drop in temperature at some time in the course of these experiments although no such fluctuation was observed. Comparisons can be made, therefore, only between Pufdi and Pufdi-and-mutant combinations within the same group of experiments.

All the Pufdi-and-mutant populations differ rather significantly from Pfd/+ in length of the developmental period. The differences are not consistently less in those populations which had a smaller wing spread, as the following comparisons of Pufdi-and-mutant populations with Pfd/+ (from data in Tables VI and VIII) indicates:

Genotype	Males:		Females:	
	Wing spread	Rate of Development	Wing spread	Rate of Development
Pfd/L^2	−	−	0	−
Pfd L^2/++	0	+	+	+
Pfd/+, M(3)w/+	0	+	0	+
gt^2/O, Pfd/+	+	+		
gt^2/gt^2, Pfd/+			+	+

O indicates a 95 per cent. chance that the Pufdi-and-mutant population is not different from the Pfd/+ population; otherwise, a plus sign is used to indicate that the Pufdi-and-mutant population exceeds Pfd/+ or a minus sign to indicate that the Pufdi-and-mutant population is exceeded by Pfd/+.

The very much longer developmental period found for Pfd/+, M(3)w/+ agrees with other published data on M(3)w stocks (Plunkett, 1926; Dunn and Coyne, 1935; Brehme, 1941). It is noteworthy that this prolongation of development by from 66 to 70 hours, which is greater than

the effect of lowering temperature by 3°, does not produce a change in wing spread.

A more rigid test of agreement between rate of development that the data in Table VIII permit is the comparative duration of development of sexes in Pfd/L^2 and Pfd L^2/++ stocks. As noted previously, these stocks differ from the others used in that they do not exhibit sexual dimorphism with regard to wing spread. Since both sexes are reared together in the same bottles, fluctuations of temperature and variations of conditions within the bottles are eliminated as sources of error and the conditions of the experiment become as nearly perfect as it is possible to make them. Consequently, any difference in rate would necessarily be attributed to a physiologic factor. Since the sexes do not differ appreciably in wing spread it might be expected that they would not differ in rate of development. The data are as follows:

MEAN DURATION OF DEVELOPMENT (IN HOURS)

Genotype	Males	Females
Pfd/L^2	212.798 ± 0.404	210.180 ± 0.461
Pfd L^2/++	209.164 ± 0.511	208.572 ± 0.507

Statistically, the rates for Pfd L^2/++ males and females may be regarded as being in fairly close agreement, whereas those of the Pfd/L^2 males and females are unquestionably different. The evidence for a rate basis for gene expression is, in this case also, not convincing.

THE EFFECT OF SEX ON RATE OF DEVELOPMENT AND WING SPREAD

The sexual dimorphism exhibited by variable mutants is pertinent to the role of rate of development in gene expression since the mean duration of development of Drosophila males is several hours longer than that of females. The sexual dimorphism is generally in the direction that would be expected from the effects of temperature, *i.e.*, males differ in the direction that low temperatures would produce. Thus males have larger eyes in Bar (Krafka, 1920; Zeleny,

1921; Margolis, 1935a) and longer wings in vestigial (Stanley, 1931; Riedel, 1935).

Reference in Tables I and VII reveals that this relationship does not obtain in the case of Pufdi. At all temperatures, males have the longer developmental periods and the greater wing spread, which are contrary results in terms of the effect of temperature independently on rate of development and wing spread.

Sources of Error

The measure of gene expression as determined in these experiments is subject to error from several sources. Prominent among them are: (1) fluctuations in temperature, (2) inaccuracies in the measurement of wing spread, (3) the possibility that the wings are not invariable in position and that wing spread is therefore not a reliable indication of gene expression, and (4) the variety of conditions which might develop within the different culture bottles and which were uncontrolled except as they may have been limited by the precautions described under experimental procedure.

The cabinets used did not control temperature more closely than within 0.75° of the desired temperature. Two developments in the course of the experiments suggest that temperature fluctuations of this magnitude might cause a fairly large error in results. The first of these is that slight differences in temperature provoke relatively large changes in the wing spread of Pufdi flies. In Table I and in Fig. 1, it is shown that between 19° and 31° wing spread increases a total of 84.236° in males and 78.896° in females. The increase is more or less evenly distributed throughout this temperature range. This means that wing spread changes 6.5°–7° for each 1° C. change in temperature. With fluctuations within the cabinets of 0.75° to either side of the desired temperature, the flies were subject to a total variation in temperature of 1.5° C., which is sufficient to produce a difference in wing spread of approximately 10°. The other development is that temperature is effective for a period of

from 6 to 8 hours only. The wing spread therefore does not reflect the average temperature at which the flies were reared, but the temperature prevailing during the brief temperature-effective period. In such a short interval, the corrective influence of compensating fluctuations is largely lost.

An error resulting from an inaccuracy in taking the measure of wing spread or from variability in the position of wings would, in either case, be revealed by comparing means calculated from separate measures of the same population. Accordingly, two measurements were made of the same sample of 100 flies. The measurements were separated by an interval long enough to allow the flies to recover completely from the first etherization. (The actual number of flies measured the second time was 97, the other 3 having been lost.) The means calculated from these measurements are:

1st measurement	69.700 ± 4.434
2nd measurement	72.441 ± 4.443
Difference	2.741 ± 6.276

The total possible error from temperature fluctuations and inaccuracies in determination in wing spread is, in most cases, sufficient to account for the differences encountered in wing spread between populations of the same genotype which had spent their entire effective period at the same temperature.

Discussion

One possible explanation for the fact that there is not the agreement between rate of development and expression of the Pufdi gene [illegible] the independent responses of these processes to temperature suggests is the lack of coincidence in time of the temperature-effective period and the time of embryonic development of the character in question. In other cases where the time of both processes is known, they occur simultaneously for at least a part of their courses, or the temperature-effective period occurs before the embry-

onic differentiation of the structure. The former is the usual situation. The eye anlage is present in the newly hatched larva, or appears soon thereafter, and the number of ommatidia approaches the number in the adult eye when about 75 per cent. of the larval period is completed (Chen, 1929; Steinberg, 1941). This period of embryonic determination includes the temperature-effective periods of the eye mutants Bar (Seyster, 1919; Driver, 1926; Margolis, 1935b) and eyeless (Baron, 1935). The wing anlage appear a few hours after hatching and the wings are completed at pupation (Chen, 1929; Robertson, 1936). This period encompasses the temperature-effective periods of vestigial (Stanley, 1931; Harnly, 1936) and short wing (Eker, 1939). The temperature-effective period for reduplicated legs occurs in the early larval period (Hoge, 1915) which is also the time of differentiation of limb buds (Robertson, 1936).

In the genes Dichaete and scute, temperature exerts its effect prior to the time of embryonic differentiation of the bristles. These appear late in the pupal period, within one day of emergence (Plunkett, 1926). The temperature-effective period of scute is larval (Child, 1935) and in Dichaete it extends over the whole developmental period (Plunkett, 1926). Presumably the bristle-destroying catalyst which Plunkett ascribes to the Dichaete gene persists until the bristle-forming processes are under way. The effect of the scute gene also is evidently persistent from its temperature-effective period to the time of bristle formation.

The Pufdi gene differs from these in that its temperature-effective period occurs neither simultaneously with nor prior to the period of wing formation, but long after all the wing-forming processes are completed. Consequently, a change in rate of development during the temperature-effective could not cause a developmental change in the already completed wing. The only structural change possible would be one caused by a gene-induced degeneration of wing tissue. This is not the case since, except for position and the occasional puffiness, the typical Pufdi wings are not different from normal wings.

The only wing-developmental process which occurs after the temperature-effective period is the unfolding and smoothing of the wings of the newly emerged adult, which occurs within the first hour after it leaves the puparium. Stern (1929) found the temperature-effective period of the Curly gene to occur during this time of unfolding. The effect of the Curly gene resembles that of Pufdi in that it does not involve structural modification of the wing. This situation, together with the example of the deferred effects of the Dichaete and scute genes, suggests that the explanation of the effect of the Pufdi gene might be sought in its influence upon this subsequent unfolding process.

Acknowledgments

It is a pleasure to acknowledge my indebtedness to Dr. A. Franklin Shull, at whose suggestion this study was undertaken, for his guidance and generous counsel throughout the course of the experiments. I owe thanks also to the Rosenwald Fund and to the General Education Board for appointment to fellowships during the period of this study.

Summary

Pufdi flies reared at temperatures of 16°, 19°, 22°, 25°, 28° and 31° C. exhibited minimum divergence of wings at 19°, and responded to increases in temperature by progressive increases in wing spread. The wing spread of wild flies was not significantly modified by temperature changes. In both Pufdi and wild populations, males had greater wing spread than females, with the doubtful exception of wild flies reared at 28°.

The temperature-effective period for Pfd/+ flies started at 19° and transferred to 31° begins in males between 6 and 8 hours before emergence of the imago, and in females between 4 and 6 hours before emergence. In both sexes, the period ended with emergence. In the 31° to 19° transfers, the effective period in both males and females begins between 8 and 10 hours before emergence and ends between 2 and 4 hours prior to that event.

Of the Pufdi-and-mutant combinations *Pfd/L²*, *Pfd L²/++*, *Pfd/+*, *M(3)w/+*, *gt²/O*, *Pfd/+*, and *gt²/gt²*, *Pfd/+*, the wing spread of the garnet-Pufdi stocks (male and female) and the Pfd L^2/++ females exceeded that of Pfd/+, and that of Pfd/L^2 males was smaller. The other combinations did not differ significantly from Pfd/+.

The Pufdi-Lobe² arrangement Pfd L^2/++ had greater wing spread in both males and females than the arrangement Pfd/L^2.

In both the Pfd/L^2 and Pfd L^2/++ arrangements, the L^2 gene cancelled the sexual dimorphism which was otherwise characteristic of wing spread.

At all temperatures, the duration of development of wild-type flies is greater than that of Pfd/+. The differences were not less at low temperatures where the two stocks were phenotypically most alike nor greater at high temperatures where they were most unlike.

The length of the developmental period (at 25° C.) of the Pufdi-and-mutant combinations bore no relationship to the differences in wing spread between these combinations.

Male Pfd/+ have longer developmental periods and greater wing spreads than females.

It was suggested that the lack of association between rate of development and the expression of the Pufdi gene might be due to the fact that the wings develop during the larval period, whereas the Pufdi gene operates during the final hours of the pupal period. Thus differences in developmental rate during the effective period of the gene would presumably not affect the already concluded wing-forming processes.

LITERATURE CITED

Baron, A. L.
1935. *Jour. Exp. Zool.*, 70: 461–490.
Brehme, K. S.
1941. *Jour. Exp. Zool.*, 88: 135–160.
Chen, Tse-Yin
1929. *Jour. Morph.*, 47: 135–199.
Child, G. P.
1935. *Genetics*, 20: 127–155.

Driver, E. C.
1926. *Jour. Exp. Zool.*, 46: 317–322.
Dunn, L. C., and J. Coyne
1935. *Biol. Zentralbl.*, 55: 385–389.
Eker, R.
1939. *Jour. Genetics*, 38: 201–227.
Harnly, M. H.
1936. *Genetics*, 21: 84–103.
Hersh, A. H.
1929. Am. Nat., 63: 378–382.
Hoge, M. A.
1915. *Jour. Exp. Zool.*, 18: 241–298.
Krafka, J.
1920. *Jour. Gen. Physiol.*, 2: 409–464.
Margolis, O. S.
1935a. *Genetics*, 20: 156–171.
1935b. *Genetics*, 20: 207–222.
Plunkett, C. R.
1926. *Jour. Exp. Zool.*, 46: 181–244.
Riedel, H.
1934. *Arch. Entw. Mech. Org.*, 132: 413–508.
Robertson, C. W.
1936. *Jour. Morph.*, 59: 351–399.
Seyster, E. W.
1919. *Biol. Bull.*, 37: 168–182.
Shull, A. F.
1938. Papers of the Mich. Acad. of Science, Arts and Letters, XXIII: 647–649.
Stanley, W. F.
1931. *Physiol. Zool.*, 4: 394–408.
Steinberg, A. G.
1941. *Genetics*, 26: 325–346.
Stern, C.
1927. *Biol. Zentralbl.*, 47: 361–369.
Zeleny, C.
1921. Am. Nat., 55: 404–411.
1923. *Biol. Bull.*, 44: 105–112.

A CASE IN RICE, *ORYZA SATIVA* L., SUPPORTING KOSAMBI'S FORMULA FOR ESTIMATING MAP LENGTHS *

N. R. BHAT

DEPARTMENT OF GENETICS, UNIVERSITY OF CAMBRIDGE

SINCE the time the linear arrangement of genes on chromosomes was established geneticists have been trying to estimate the actual lengths of chromosomes or parts thereof for two reasons. First, map lengths are useful to infer the relative size of a chromosome containing any particular factors. Second, the knowledge of the relation existing between recombination fractions and linear intervals would enable expectations to be made of gametic frequencies in hybrids segregating in several factors.

As is well known, recombination fraction, the observable index of exchange between homologous chromosomes, is not suitable for measuring map-distances, when they are larger than about 10 c.o.v., due to the possibility of the occurrence of multiple crossovers. For the same reason, the intervals measured by recombination fractions are not additive. Indeed, in such measurement the length of a chromosome must continue to increase as more and more factors are discovered in it. However, no better unit has been discovered so far for the purpose.

Recently, Kosambi (1944) has proposed a formula for estimating map lengths from recombination fractions, the relation assumed between the two being, $2y = \tanh(2x)$, where y is the recombination fraction and x the map distance. But, the formula is empirical and, therefore, requires to be tested in a variety of cases before being adopted

* Section VII of the thesis submitted for the degree of Ph.D. to the University of Cambridge.

for general use. If it is found to be widely applicable, it may prove a very useful tool in genetic work.

A number of animals and plants possess fairly large chromosome complements. Some of these chromosomes may be quite long. For these reasons, the study of linkage groups in such organisms is difficult, particularly when the factors discovered in them are limited in number. In such cases the probability of locating even a few factors on a single chromosome is small. In addition, when the number of individuals bred is not large enough for discrimination, loosely linked factors may be mistaken as independent. This happened to be the case in the example chosen for this paper. Kosambi's formula, when applicable, would indicate whether it is likely to be the one or the other. Moreover, the lengths of intervals estimated by using the formula would remain almost unaltered, irrespective of the magnitude of recombination, even when new factors are discovered in them. On the other hand, additional factors are expected to substantiate the previous estimates.

Till now the formula has been used with success in three different cases. Fisher (1946b) applied it first in the test for linkage between the factors, *W*, *s* and *hr* in the house mouse. The present author found it applicable to Punnett's data on the "B" chromosome in the sweet pea. Rao (*Heredity*, in press) tried it to work out the backcross results of De Winton and Haldane (1935) in *Primula sinensis* L. He has also proved that when the formula is used, the estimates made with two factor segregations are almost as accurate as those based on the simultaneous segregation of three or more factors. The present example is chosen as an additional case supporting the formula.

Materials and Methods

Jodon (1940) discovered a chlorophyll mutation, virescent, in a strain of rice, No. 2912-A-21. Since the strain was breeding true for four other characters, he utilized the mutation for studying its linkage relations with them. For this purpose, he crossed the strain with another, No. C.I.

4630, which was pure for all the contrasting characters. The description of the two strains, as given by him, is reproduced below along with their genotypes with respect to the factors investigated.

Strain No.	Character	Genotype
2912 A-21	Virescent seedlings	v, v
	Normal arrangement of florets	cl, cl
	Nonglutinous grains	Gu, Gu
	Colorless apiculus	as, as
	Late maturity	Fl, Fl
C.I. 4630	Nonvirescent seedlings	V, V
	Clustered florets	Cl, Cl
	Glutinous grains	gu, gu
	Purple apiculus	As, As
	Early maturity	fl, fl

The F_1 plants were non-virescent and had normal arrangement of florets and non-glutinous grains. They were late to flower and the apiculi of their spikelets were purple. Thus, their genotype was Vv, Clcl, Gugu, Asas, Flfl. From these he raised 890 F_2 plants for study. The segregation of individual factors among them was as below:

V	v	Cl	cl	Gu	gu	As	as	Fl	fl	Total
691	199	673	217	673	217	665	225	678	212	890

As can be seen from the above frequencies there was a satisfactory segregation for three out of the five factors, that for the other two, particularly V-v, being poor. On studying them two by two, he found evidence of linkage in nine out of the ten combinations, but not in the one for *gu* and *cl*, χ^2 for association being only 2.067. The recombination fractions were estimated by the product formula, a method appropriate to such data involving deficiencies of recessives. They were as given in Table 1. For the reason that χ^2 for linkage was not significant, the estimate for *gu-cl* was not given but was called independence.

TABLE 1

PERCENTAGES OF RECOMBINATION BETWEEN THE FIVE FACTORS STUDIED IN THE CROSS BETWEEN THE STRAINS, 2912 A-21 AND C.I. 4630

Factors	v	cl	gu	as	fl
v	....	34.5	40.5	24.5	7.5
cl	...	.....	independence	42.5	40.5
gu	....	.....	.....	22.5	41.5
as	....	.....	.....	.....	30.0
fl	....	.....	.....	.....	.....

A chromosome map was drawn with the above estimates. There is a great deal of ambiguity about the factor, *fl*, which has been discussed at length by the author. Particularly, its recombination of 30 per cent. with *as* and 40.5 per cent. with *cl* are irreconcilable. For this reason the factor had to be left out of consideration altogether.

In view of Rao's (*Heredity*, in press) finding mentioned above, it was considered satisfactory to make estimates with data on only two factor segregations. As they have not been given in the original paper, they were reconstructed from the given estimates. In the case of *gu-cl* combination they were derived from the χ^2 for association which seems to have been calculated by the contingency table method.[1] They are given in the Appendix. These frequencies were utilized to test Kosambi's formula. Since the frequencies of the middle two classes do not differ significantly, the maximum likelihood method should not give estimates very different from those made by the product formula. As the former method is more convenient for scoring and combining information, it was used in preference to the latter.

The procedure of estimation is much the same as was adopted in the case of Punnett's "B" chromosome in the sweet pea (Bhat, 1947), *i.e.*, to make the estimates for individual segments by scoring the two factor segregations and then to combine the scores and information to obtain the final estimates. The formulae required to be used for combining data on three segments are given below as they may be useful for practical workers.

Estimate of (a segment containing three smaller ones, *e.g.*)

$$y_{123} = \frac{y_1 + y_2 + y_3 + 4y_1y_2y_3}{1 + 4y_1y_2 + 4y_1y_3 + 4y_2y_3}$$

Factor to obtain the contribution (of a segment consisting of three smaller ones to one of the latter, *e.g.*)

of y_{123} to y_1 is $\frac{y_{123}}{y_1} = \frac{(1 - 4y_{23})^2}{(1 + 4y_1y_{23})^2}$

[1] Data on the simultaneous segregation of all the five factors were very kindly supplied, later, by Jodon. The reconstructed frequencies were found to be the same as the actual frequencies.

Starting with the original estimates, *i.e.*, y_1(gu-as) = .225, y_2(as-v) = .245, y_3(v-cl) = .345 and by using the appropriate formulae, the first approximation of estimates was made for y_{12}, y_{23} and y_{123}. All the estimates along with scores for single segments are set out in Table 2.

TABLE 2

ESTIMATES REPRESENTING THE FIRST APPROXIMATION AND SCORES FOR SINGLE SEGMENTS

Single segments	Estimates	Estimates of segments made of two adjoining ones	Estimates of the segment made of three segments	Scores for single segments
y_1	.225	.38509		− 8.1953742
y_2	.245	.44092	.47674	− 6.6073962
y_3	.345			+ 18.7927614

The scores for single segments given in the last column of Table 2 consist of their own scores plus the contribution from the larger segments wherein they are included. Thus, the score for y_1 is derived from y_1, y_{12} and y_{123} and that for y_2 from y_2, y_{12}, y_{23} and y_{123}, and so on. For example, the score − 6.6073962 for y_2 is derived as follows:

From segment	Score	Factor	Contribution to y_2
y_2	− 4.5153777	.....	− 4.5153777
y_{12}	− 21.9327528	.5353713	− 11.7421664
y_{23}	+ 24.3096383	.2925978	+ 7.1129467
y_{123}	+ 21.2154049	.1195924	+ 2.5372012
			− 6.6073962

The information for the three segments was similarly built up by using the squares of the factors in each case. Moreover, the product terms, I_{12}, I_{23} and I_{13} were also calculated. The factors for these are the products of the factors used in the case of the first three segments, and each of the above was arrived at as illustrated in the case of I_{12} below.

From segment	Information	Factor for y_1	Factor for y_2	I_{12}
y_{12}	1133.5329	.5101299	.5353713	309.57792
y_{123}	1452.05397	.1139638	.1195924	19.79033
				329.36285

The information thus derived is arranged in the form of the following matrix:

1302.00548	329.09689	28.70536
329.09689	3855.04077	255.17971
28.70536	255.17971	2611.89874

The reciprocal matrix obtained by adopting the "c" matrix method (Fisher, 1946a, pp. 158) was:

$\overset{3}{+07849953}$	$\overset{4}{-06687485}$	$\overset{5}{-02090888}$
$\overset{4}{-06687485}$	$\overset{3}{+02667862}$	$\overset{4}{-02532976}$
$\overset{5}{-02090888}$	$\overset{4}{-02532976}$	$\overset{3}{+03853608}$

which, when multiplied by the scores of single segments (with signs reversed), gave the following adjustments to the estimates:

y_1	y_2	y_3	
$\overset{2}{+06030755}$	$\overset{2}{+01690714}$	$\overset{2}{-07426493}$	adjustments
.23103	.24669	.33757	adjusted estimates

On the basis of these adjusted estimates new estimates were made for y_{12}, y_{23} and y_{123} by using Kosambi's formula. They were .3890319, .4382715 and .4763659, respectively. The scores for the single segments at the new values of estimates (derived by adopting the procedure explained above) were:

y_1	y_2	y_3
+0.1658208	+0.5139065	−0.3660751

As the new scores for all the three segments were almost zero, no further adjustments were necessary and the estimates given above were themselves final. In order to calculate their standard errors it was necessary to derive the inverse of their information matrix. The inverse matrix is given below:

$\overset{3}{+07561936}$	$\overset{4}{-06741996}$	$\overset{5}{-01560406}$
$\overset{4}{-06741996}$	$\overset{3}{+02600764}$	$\overset{4}{-02419804}$
$\overset{5}{-01560406}$	$\overset{4}{-02419804}$	$\overset{3}{+03573091}$

The variances of the three primary estimates are given by the three figures along the leading diagonal in the inverse matrix. In this case they were:

$$V_{y_1} = \overset{3}{07561936} \qquad V_{y_2} = \overset{3}{02600764} \qquad V_{y_3} = \overset{3}{03573091}$$

Those for a segment, *e.g.*, y_{12}, made up of two adjacent segments were calculated by the formula,

$$Vy_{12} = \eta_1^2(Vy_1) + \eta_2^2(Vy_2) + 2\eta_1\eta_2 \cdot C(y_1y_2)$$

where

$$\eta_1 = \frac{\delta y_{12}}{\delta y_1};\ \eta_2 = \frac{\delta y_{12}}{\delta y_2} \text{ and } C = \text{cofactor of } I_{12} \text{ (in this case } -\overset{4}{0}6741996).$$

Similarly,

$$Vy_{123} = \eta_1^2(Vy_1) + \eta_2^2(Vy_2) + \eta_3^2(Vy_3) + 2\eta_1\eta_2 \cdot C(y_1y_2) + 2\eta_1\eta_3 \cdot C(y_1y_3) + 2\eta_2\eta_3 \cdot C(y_2y_3)$$

Map lengths in centimorgans corresponding to the six estimates were found by referring to Fisher and Yates (1938, Table VII, Transformation of "r" to "z"). Their standard errors were estimated by the formula,

$$\text{S.E. } (x) = \frac{\text{S.E. } (y)}{1 - 4y^2}$$

where y is the estimate of any particular segment and x the map length.

The Results

The final results are set out in Table 3.

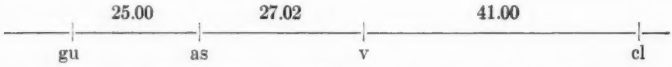
TABLE 3

Recombinations Per Cent, Map Lengths and χ^2s to Test Agreement With Kosambi's Formula of the Six Segments in the Linkage Group Established by Jodon (1940)

Segment	Recomb. per cent.	Maplengths in Centimorgans	χ^2	P
gu-as	23.10 ± 2.75	25.00 ± 3.50	0.042	
as-v	24.67 ± 1.61	27.02 ± 2.13	0.000	
v-cl	33.76 ± 1.89	41.00 ± 3.47	0.061	
gu-v	38.90 ± 1.50	52.02 ± 3.80	0.235	
as-cl	43.83 ± 0.91	68.02 ± 3.93	0.208	
gu-cl	47.64 ± 0.46	93.02 ± 4.99	0.284	
		Total	(3)0.830	> .8

It will be noticed that the standard errors for all the estimates are low enough to make the latter reliable. The χ^2 for 3 d.f. (six for estimates minus three for the segments) is only 0.83, the probability of which is more than 80 per cent. So, the agreement with Kosambi's formula is very good and justifies its application. The map lengths are all additive, as they are expected to be, those for the larger segments being the totals of the smaller component seg-

ments. A map of the chromosome in terms of map distances is given below:



Incidentally, it was thought worth while to see if indications could be obtained about the position of the factor *fl*, from the observed frequencies of the sixteen phenotypes for the simultaneous segregation of the other four factors. They are given in Table 4 along with the expectations based on the final estimates assuming triple crossovers to be nil. The expectations were made by the method given by Rao (*Heredity,* in press). The distribution of late (Fl) and early (fl) plants in each of the classes has also been shown.

The total observed frequencies agree fairly well with the expectations; but the distribution of late and early plants is not satisfactory. Since *fl* is expected to be either in the *as-v* segment or in the *v-cl* segment, a comparison of the proportion of the triple recessive (*as, fl, v* and *v, fl, cl*) plants should suggest the position of *fl*. Actually, however, only one plant of the former type has occurred amongst 118 *as-v* plants and also one in a total of 86 *v, cl* plants.

TABLE 4

FREQUENCIES OF THE SIXTEEN PHENOTYPES FOR THE SEGREGATION OF THE FOUR FACTORS AND THE DISTRIBUTION OF LATE AND EARLY PLANTS IN THEM

Phenotypes	Expected total	Total	Observed Fl	fl
Gu as v cl	49.11	50	49	1
gu As V Cl	149.71	148	90	58
gu as v cl	4.03	2	2	0
Gu As V Cl	313.74	325	221	104
gu As v cl	11.47	7	7	0
Gu as V Cl	75.06	85	69	16
Gu as v Cl	69.40	60	60	0
gu As V cl	35.00	35	21	14
Gu As v cl	33.02	27	27	0
gu as V Cl	4.12	2	2	0
gu As v Cl	14.45	16	16	0
Gu as V cl	17.06	19	17	2
gu as v Cl	3.72	6	6	0
Gu As V cl	72.81	76	59	17
Gu As v Cl	37.30	31	31	0
gu as V cl	0.00	1	1	0
	890.00	890	678	212

Parents: $\frac{\text{Gu, as, v, cl, (Fl)}}{\text{Gu, as, v, cl, (Fl)}}$ and $\frac{\text{gu, As, V, Cl, (fl)}}{\text{gu, As, V, Cl, (fl)}}$

Recombination percentages: Gu-as 23.10; as-v 24.67; v-cl 33.76; gu-v 38.90; as-cl 43.83; gu-cl 47.64

The plant in question may have occurred even by chance. The situation has become unsatisfactory, since both the factors crossing over with (recessive) *fl* in the two segments happen to be recessive. Thus, no indications could be obtained regarding the position of *fl* on the chromosome.

Summary

The validity of Kosambi's formula to estimate map lengths is tested with Jodon's (1940) data on the segregation of four factors, *gu, as, v* and *cl,* located in a single chromosome in rice. The data were found to agree very well with the formula. The method of testing is briefly described. A map of the chromosome in centimorgans is given on the basis of the estimates made by applying the formula. The situation regarding the factor *fl* is discussed.

Acknowledgments

My thanks are due to Dr. N. E. Jodon, associate agronomist, Rice Experiment Station, Crowley, Louisiana, for very kindly supplying the data on the simultaneous segregation of the five factors.

I am highly obliged to Professor R. A. Fisher, F.R.S., for his criticism and to C. R. Rao, of the Duckworth Anthropological Laboratory, Cambridge, for help in making the expectations of phenotypes for the four factor segregations.

LITERATURE CITED

Bhat, N. R.
1947. *Jour. Genet.,* 48: 343–358.
Fisher, R. A.
1946a. "Statistical Methods for Research Workers." 10th ed. Edinburgh. Oliver and Boyd.
1946b Am. Nat., 80: 568–578.
Fisher, R. A., and F. Yates
1938. "Statistical Tables." Edinburgh. Oliver and Boyd.
Jodon, N. E.
1940. *Jour. Am. Soc. Agron.,* 32: 342–346.
Kosambi, D. D.
1944. *Ann. Eugen., Camb.,* 12: 172–175.
Rao, C. R.
(In press). "On Methods of Scoring Linkage Data Giving the Simultaneous Segregation of Three Factors." *Heredity.*

APPENDIX

F_2 Phenotypic Frequencies Derived from Single Factor Segregations by Utilizing the Estimates Made by Jodon (1940)

Mating Type	Phenotypes				Total
Repulsion	Gu, As 459	Gu, as 214	gu, As 206	gu, as 11	890
Coupling	As, V 584	As, v 81	as, V 107	as, v 118	890
Coupling	V, Cl 560	V, cl 131	v, Cl 113	v, cl 86	890
Repulsion	Gu, V 505	Gu, v 168	gu, V 186	gu, v 31	890
Coupling	As, Cl 520	As, cl 145	as, Cl 153	as, cl 72	890
Repulsion	Gu, Cl 501	Gu, cl 172	gu, Cl 172	gu, cl 45	890